THE PATHOPHYSIOLOGICAL CONSEQUENCES OF AN IN UTERO HYPOXIC INSULT ON THE FOETAL RAT BRAIN

by

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DECLARATION

In accordance with the requirements of the University of Edinburgh regulation 3.4.7, this thesis has been composed by myself and the work presented herein is my own.

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ABSTRACT

The occurrence of one or more hypoxic-ischaemic episodes during the perinatal period is believed to contribute to a range of non-progressive neuronal deficits in the human neonate. There are presently no widely accepted animal models of this clinical condition that allow the study of the pathophysiology of perinatal hypoxia. The aim of this study was to investigate the effects of an *in utero* hypoxic insult on the near-term foetal rat brain.

An hypoxic insult of 10, 20 or 30 minutes was created by clamping the utero-placental vessels of one uterine horn in an anaesthetised pregnant Lister hooded rat at 22 days gestation. After completion of the occlusion period the dam was killed and the foetal rats were delivered by Caesarian section and resuscitated. Alternatively the clamps were removed, the uterine horn placed back inside the dam and the wound sutured. After a recovery period of 20 minutes, during which time the dam was not anaesthetised, the dam was killed and the foetal rats were delivered by Caesarian section and resuscitated.

Biochemical analysis of homogenised and deproteinised brain tissue samples from the neonatal rats showed a level of metabolic deficit typically associated with irreversible neuronal necrosis in adult models of hypoxic-ischaemia. The level of brain tissue lactate in rats subjected to a 30 minute clamp without recovery was 19.77 $\pm$ 0.8nmols/mg brain tissue whereas in control rats the level of lactate was 7.98 $\pm$ 0.2nmols/mg brain tissue ($p < 0.001$). Brain tissue ATP declined by more than 80% from 2.67 $\pm$ 0.2nmols/mg brain tissue in control rats to 0.59 $\pm$ 0.1nmols/mg brain tissue in hypoxic rats ($p < 0.001$). Other metabolites also showed changes consistent with a major hypoxic insult.

Hypoxic neonatal rats and their littermate controls were cross-fostered onto nursing Cob-Wistar dams to enable measurements during the postnatal period. Assessment of the increase in weight and head-to-tail length of hypoxic and control rats from pnd0-pnd42 showed no significant difference between the two groups. Activity ratings and development of the righting reflex were also similar for the two groups.

Despite the metabolic deficit indicated by the biochemical data histological analysis showed a lack of gross lesions or widespread neuronal necrosis, although there was some indication of an increase in the number of dead or dying neurons around the CA3 region of the hippocampus and the hind brain nuclei. The brains of adult rats that had been subjected to an *in utero* hypoxic insult before birth exhibited no gross neuropathological changes. However, analysis of the hippocampal CA1 width of the adult rat brains showed that hypoxic CA1 regions were significantly narrower (3-6 %, $p < 0.05$) than control CA1 regions. This reduction might reflect changes in hippocampal neuronal density, cell size or alterations in dendritic outgrowth.

A place navigation task was used to assess spatial memory in the rats at 3 months of age. The rats were trained to find a hidden platform in a 2m diameter water maze made opaque by the addition of powdered milk. Acquisition of the task was achieved at a similar rate by both the control and the hypoxic rats. In contrast, the performance of rats with hippocampal lesions induced by injection of ibotenic acid was significantly impaired ($p < 0.001$). In a transfer test control rats spent 56.59 $\pm$ 3.8% of the testing period in the training quadrant whereas hypoxic rats spent 51.13 $\pm$ 4.7% of the testing period in the training quadrant ($p > 0.05$). Hippocampally lesioned rats spent only 31.28 $\pm$ 6.9% of the testing period in the training quadrant which was significantly less than both the control and the hypoxic rats ($p < 0.001$).

This study has shown that despite a significant metabolic deficit those rats which survive an *in utero* hypoxic insult suffer only limited and discrete neuropathological changes and no apparent long-term physiological or cognitive deficits. These results suggest that the near-term foetal rat brain has a high level of resistance to the long term consequences of a severe hypoxic insult.

ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AOCC	Agonist Operated Calcium Channels
AP7	2-Amino-7-phosphonoheptanoate
APV	2-Amino-5-phosphonovalerate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BDNF	Brain Derived Neurotrophic Factor
CBF	Cerebral Blood Flow
CPP-ene	3-((-)-2-Carboxypiperazin-4-yl)propenyl-1-phosphonate
CSF	Cerebrospinal Fluid
CT	Computerised Tomography
DNA	Deoxyribonucleic Acid
E	Gestational age
EEG	Electroencephalogram
FFA	Free Fatty Acid
GABA	γ-Aminobutyrate
GFAP	Glial Fibrillary Acidic Protein
IP_3	Inositol triphosphate
LDH	Lactate Dehydrogenase
MCA	Middle Cerebral Artery
MINS	Minutes
MK-801	(+)-5-methyl-10,11-dihydro-5h-dibenzo [a,d] cyclohepten-5,10-imine
MRI	Magnetic Resonance Imaging
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide-reduced form
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoxylbenzo(F)quinoxaline
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
PCr	Phosphocreatine
PET	Positron Emission Tomography
PLA_2	Phospholipase A_2
pnd	postnatal day
PVL	Periventricular leukomalacia
VSCC	Voltage Sensitive Calcium Channels

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CONTENTS

CHAPTER 1: INTRODUCTION

CHAPTER 2 : MATERIALS AND METHODS

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 General Development of the Central Nervous System

A knowledge of the fundamental development of the central nervous system (CNS) is an essential prerequisite to any research investigation which involves some element of perinatal stress or trauma. The organisation and interconnections within the CNS are to a great extent predetermined by genetic information. That information is limited to around 10^5 genes in mammals yet it is postulated that around 10^{15} interconnections exist in the mammalian CNS (Kandel and Schwarz, 1985). It is therefore reasonable to suggest that epigenetic factors may be involved in the designation of function and activity within the CNS. The extent to which non-genetic factors contribute to the development of the CNS is still a matter of great debate and speculation but it is believed that trauma or stress, whether environmental or experimentally induced, during the critical periods of development can lead to irreversible neuronal deficits.

The initial phases of development are common to all organisms and differ only in the time course at which they proceed. Eight major phases exist in the development of the CNS (Cowan, 1979). The first phase is one of induction, in which a population of non-specific cells within the ectodermal region of the foetus become irreversibly committed to form the neural plate, the precursor of the CNS. Sequential release of glycoprotein messenger molecules from the underlying mesoderm is responsible for this induction of the ectoderm to form neural tissue. The sequential pattern of induction is such that the forebrain region is induced before the spinal cord region and this sets the pattern for regional development of the CNS (Cowan, 1979).

The neural plate invaginates into the neural groove which then closes to form the neural tube (Jacobson,1978; Purves and Lichtman, 1985; Kandel and Schwarz, 1985). The cavity of the neural tube forms the ventricular system whereas the neurons and glia are derived from the single cell epithelial layer around the perimeter of the tube which is referred to as the neuroepithelium.

Once the neural tube is completely closed the second phase of development, which is one of proliferation, begins. Mitosis occurs in germinal zones along the length of the tube to form specific regions of growth. Each CNS region has differing numbers of divisions and at certain specific time points the stem cells in a particular region cease to divide and migrate out of the

epithelium to form a second cellular layer, referred to as the intermediate layer. The majority of 'neuronal' cells are incapable of any further division but glial cells can synthesise DNA throughout life.

The point at which a neuron ceases to synthesise DNA is referred to as the 'birth date' and labelling studies with [^{3}H]-thymidine have identified this time-point for certain cell populations (Cowan, 1979). As a general rule larger neurons, in particular those which must extend long processes, are generated before smaller neurons. For example, the cells which occupy the deepest cortical layers are generated before those which occupy the superficial layers. The first supporting glial cells of each region generally appear at the same time as the first neurons.

The third phase of development involves migration of neurons from their point of origin to their final destination. Although the majority of migrating neurons are post-mitotic, there are some exceptions such as the cells of the subventricular zone. The cells of this particular zone proliferate after migration to form the small neurons and glial cells of the basal ganglia and other deep nuclei of the cerebral cortex. Migration proceeds at the slow pace of around 0.1mm per day and in many regions is guided by the radial processes of specialised glial cells; for example, the Bergmann astrocytes which guide the granule cells of the cerebellar cortex (Kandel and Schwarz, 1985).

The fourth stage is one of organisation or aggregation during which cell populations that have reached their final destination group together, like with like, to form nuclei or laminae. The cells adhere in precise configurations and often with specific orientation within the group. For example, the pyramidal cells of the cerebral cortex are always aligned such that the apical dendrites are directed toward the outer surface.

Phase five is referred to as a period of cytodifferentiation during which each neuron develops the specific intracellular and membrane characteristics by which it is identified in the adult CNS.

The next three phases of development involve many 'critical periods' which vary greatly among species but are alike in that experimental interference during these periods can produce irrevocable damage/changes to the organisation and functioning of the adult CNS. These stages are the most accessible for investigation as they occur in the final days or weeks of gestation and the first weeks of postnatal life.

The sixth phase involves the formation of specific connections with other neurons beginning with axonal and dendritic outgrowth. Axons grow by way of a growth cone at their tip and typically will grow along a surface of high adhesiveness. Many axons also have the molecular capacity to respond to chemical and structural guidelines along the length of their paths. The release of trophic substances such as nerve growth factor (NGF) by the target cell also provides guidance for the axonal path (Levi-Montalcini, 1952; Levi-Montalcini, 1975). The majority of neurons extend many more processes than are required or can be maintained and so must retract these excess processes during maturation.

Upon reaching the target region each axon must form specific synaptic connections with the correct target cells and as neuronal phenotype has already been specified there can be two-way interactions between pre- and post-synaptic cells to verify the specificity of the connection. Each neuron makes many more connections onto other neurons than are found in the adult CNS; for example, the Purkinje cells of the cerebellum receive input from several climbing fibres in early life but from only one in adult life. A later phase of synapse elimination rationalises the number of connections, sometimes to just one synapse.

The specificity with which cell to cell connectivity occurs in the CNS is well illustrated by the experiments of Sperry and colleagues using optic nerve regeneration in the goldfish (Attardi and Sperry, 1963). The optic nerve of one eye was severed before the point of crossover, at the optic chasm, and in some experiments this was combined with removal of part of the retina. Histological analysis was performed between 17 and 25 days after the section. The optic nerve fibres regenerated in an ordered and predictable pattern following the same topographic representation which had previously existed in the intact projection. The regenerating axons aligned to their destined path well before division of the fibres into the medial or the lateral tract. When a particular area of the retina was removed the corresponding

area of the optic tectum was not reinnervated, for example removal of the dorsal retina led to a lack of reinnervation of the lateral tectum. The precision with which the optic fibres regenerated led Sperry to formulate the chemoaffinity hypothesis which proposes that chemical differentiation among axons allows for the peripheral selection of pathways and target neurons during outgrowth (Attardi and Sperry, 1963).

The seventh and eighth phases of development are the phases that critically control the highly ordered system of interconnections within the CNS. The number of neurons is reduced to the final adult population by a period of programmed or naturally occurring cell death during which 15-85% of the initial population die (Clark, 1985; Oppenheim, 1991). The time course for this cell death differs among species; in the chick embryo cell death occurs in the cerebellar nuclei during embryonic life; in the mouse cell death occurs in the lateral geniculate nucleus, resulting in the loss of 30% of neurons, between pnd 5 and pnd 30; in the rat 45% of the neurons in the retinal ganglion die between pnd 5 and pnd30; and in the macaque monkey 16% of the neurons in the striate cortex die between pnd 1 and adult (Clarke, 1985).

The cell death usually occurs concurrently with synapse formation and is believed to be modulated by competition between populations of axons vieing for a limited number of functional contacts (Okado and Oppenheim, 1984; Clarke, 1985) and by the retrograde transport of trophic factors, such as NGF and BDNF, from the target region (Levi-Montalcini, 1975; Oppenheim, 1991).

A final phase of synapse and process elimination reduces the number of connections innervating neurons. The purpose of overpopulation during development is not known but cell death serves to match the numbers of cells in communicating populations, and synapse elimination ensures that communication is ordered and precise (Cowan, 1979).

During the fine tuning of the CNS late in development there is an element of plasticity within certain populations of neurons. The development of ocular dominance columns in area 17 of the visual cortex in cats and monkeys has been catalogued (Levay *et al*, 1978; Levay *et al*, 1980). These columns, particularly in the deeper cortical layers 4, 5 and 6, are divided such that the groups of cells in each column respond to only one eye. Monocular deprivation in kittens or new born monkeys leads to an expansion of the columns receiving input from the normal eye but

only if the deprivation occurs during the 'critical period' (Hubel and Wiesel, 1970; Hubel *et al*, 1977). This critical period occurs in the first few weeks of postnatal life and during this time the visual processes retract into the sharply defined columns whose widths depend on the competition between terminals receiving input from each eye. Monocular deprivation alters the pattern of visual responses because the cells responding to the input from the closed eye are at a competitive disadvantage to the cells responding to visual input from the open eye. It is interesting to note that the cells responding to the closed eye do not die instead they become irreversibly unresponsive to visual input.

Recently the cellular basis of this plasticity has been investigated through the pharmacological manipulation of excitatory amino acid receptors, in particular the N-methyl-D-aspartate (NMDA) receptor. The physiological responses of neurons in area 17 of the visual cortex can be reduced by ionophoretic application of the competitive NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) (Fox *et al*, 1989). Superficial cortical layers 2 and 3 are susceptible to a 30% reduction of responsiveness by application of APV in cats of all ages. In contrast the visual responsiveness of cortical layers 4, 5 and 6 is only vulnerable in 3 week-old kittens, at which time a 30% reduction in responsiveness is possible through the action of APV (Fox *et al*, 1989).

Long-term exposure to APV by the introduction of minipumps into the striate cortex of kittens can block the effects of monocular deprivation (Kleinschmidt *et al*, 1987). The shift in ocular dominance caused by monocular deprivation is prevented in the presence of APV but the action of APV severely impairs the spatial orientation of the kitten's visual responses. These experiments suggest a role for the NMDA class of glutamate receptor during the developmental modification of the visual responses, and also in the maintenance of certain properties of vision. In deeper layers of the visual cortex (4, 5 & 6), however, the role of the NMDA receptor would appear to recede with maturation.

All of the above experiments demonstrate the ability to manipulate the development and organisation of the CNS at certain critical stages of an animal's growth. This is of interest to any investigation which seeks to explore the effects of trauma on the CNS during perinatal development.

1.2 Hypoxic-Ischaemic Brain Injury in Human Infants

A hypoxic-ischaemic insult to the foetal brain during the perinatal period may result in serious, sometimes fatal, neurological injury. Up to 40% of non-progressive neurological deficits are thought to be related to a hypoxic-ischaemic insult during the critical perinatal period (Hill and Volpe, 1981; Levene, 1987; Towbin, 1970). Hypoxia, which causes reduced blood oxygen levels (hypoxaemia), or ischaemia, which involves reduced blood supply to the tissues, rarely occur in isolation but when occurring together, as in asphyxia, one or the other tends to predominate. The causes of such insults are multifarious ranging from placental defects to birth trauma as the result of a difficult forceps delivery. The pathological consequences of each type of injury are quite different and when hypoxemia occurs together with ischaemia distinct patterns of damage are seen. The early neurological manifestations of hypoxic-ischaemic injury can be as limited as a mild encephalopathy or as severe as deep coma and eventually death (Hill and Volpe, 1981; Levene, 1987).

1.2.1 Common Causes of Hypoxic-Ischaemic Encephalopathy

The common factor in hypoxic-ischaemic injury is usually one, or several, periods of intrauterine asphyxia. Intrauterine asphyxia may be caused by maternal circulatory problems, maternal haemorrhage due to placenta praevia or abrupto placenta, a prolapsed umbilical cord or a cord wrapped tightly around the infant's neck. Asphyxia may also occur during parturition, particularly if there has been a previous intrauterine insult. Traumatic birth injury related to prolonged labour, breech presentation or a difficult forceps delivery is another contributory factor to intrapartum asphyxia. Hill and Volpe (1981) state that 90% of cases of hypoxic-ischaemia are due to intrauterine asphyxia of which only 25% are related to traumatic events during parturition. Any consequences of intrauterine or intrapartum asphyxia can be further complicated by the influence of prematurity, maternal undernutrition, multiple births and growth retardation of the foetus (Larroche, 1985; Hill and Volpe, 1981; Levene, 1987; Volpe, 1987).

1.2.2 Diagnosis and Incidence of Hypoxic-Ischaemic Encephalopathy

The collation of details of the incidence of hypoxic-ischaemic brain injury is greatly complicated by the use of a range of diagnostic methods and particularly by the inclusion of data relating to both premature and full-term infants. There are several accepted forms of diagnosis of neonatal asphyxia and the following section will discuss some of them.

a) The APGAR Score

This is a ten point scale based on the observation of neonatal respiration, reflexes, muscle tone, heart rate and colour (Apgar, 1953). A score of zero is given for a total lack of any vital signs of life. An APGAR score of 0-3 at one and five minutes after birth is taken as an indication of hypoxic-ischaemia or neonatal asphyxia (Nelson and Ellenberg, 1981). The incidence of perinatal asphyxia, using this method of diagnosis, is between 8 and 11.4/1000 live born births per annum (Nelson and Ellenberg, 1981; Palme and Ericsson, 1986). Although limited in its usefulness the APGAR score can give a rough indication of the occurrence of asphyxia but not the duration of the insult.

b) Positive - Pressure Ventilation

The need for positive pressure ventilation in the first 5-20 minutes of life has also been taken as evidence of an hypoxic-ischaemic insult. Brown *et al* (1974) combined this method of diagnosis with a low APGAR score to describe an incidence of asphyxia of 54/1000 live-born births per annum. MacDonald *et al* (1980) using this criterion show an overall incidence of 11.6/1000 live-born births but asphyxia is far more common in premature infants (90/1000 live-born births) in comparison with full-term infants (8/1000 live-born births). Due to the association of neonatal apnoea with disorders other than perinatal asphyxia, eg congenital CNS malformations, this criterion is no longer considered to be valid.

c) Hypoxic - Ischaemic Encephalopathies

A far more detailed and accurate diagnosis of an hypoxic-ischaemic insult to the neonatal brain is provided by clinical observation of the neurological signs using a 3 point scale to grade the severity of the hypoxic-ischaemic encephalopathy as mild, moderate or severe (Sarnat and Sarnat, 1976; Finer *et al*, 1983; Fenichel, 1983).

A mild (Grade I) encephalopathy usually lasts for around 24 hours with complete resolution and total recovery by 48 hours. A moderate (Grade II) encephalopathy can last for between 2 and 14 days but a complete recovery is likely even if neonatal seizures develop.

Severe (Grade III) encephalopathies are rare and usually have the worst prognosis. The symptoms can last for hours to weeks and the infant is stuporous, sometimes comatose, flaccid and exhibits very few, if any, reflexes. Seizures occur in around half the cases within the first 12 hours but evidence of the seizures may only be seen on the EEG. Often between 12-24 hours the level of consciousness improves but it tends to deteriorate after this until around 72 hours after birth by which time any signs of recovery must have appeared if the infant is to survive. Levene and colleagues (1985) used this grading system to record an incidence of perinatal asphyxia in 6/1000 live-born births per annum. However, only 1/1000 live-born births were in the severe category.

A variety of other methods can be used to diagnose perinatal asphyxia including blood gas and blood pH measurements, analysis of biochemical markers such as lactate or hypoxanthine and detection of foetal stress through foetal heart rate monitoring. The value of these techniques is disputed particularly in light of the recent advances in non-invasive techniques of neonatal monitoring such as real-time and Doppler ultrasound, magnetic resonance imaging (MRI), spectroscopy, computerised tomography (CT) and positron emission tomography (PET). Real-time ultrasound and CT scans are extremely valuable in the detection of cerebral haemorrhage and Doppler ultrasound has been found to correlate reduced cerebral blood flow velocity with a high risk of morbidity (Volpe, 1992; Rosenkrantz and Zalneraitis, 1991). The excellent resolution of MRI is useful in the accurate detection of the location and the extent of the neuropathological lesions (Barkovich and Truwit, 1990; Goplerud *et al*, 1993). Unfortunately these non-invasive techniques tend to be expensive, involve moving the infant to the appropriate testing location and some involve exposure to radiation.

The accurate diagnosis of birth asphyxia has been a matter for recent debate particularly since many features usually associated with birth asphyxia are also frequently observed in infants born with congenital CNS malformations such as hydrocephalus (Naeye *et al*, 1989, Bedrick, 1989; Nelson and Leviton, 1991). These features include the presence of meconium in

the amniotic fluid, neonatal apnoeic episodes, noenatal seizures and persistent neurological abnormalities (Naeye *et al*,1989). The problems with detection of the cause of a neurological deficit can lead to the long-term disability being falsely ascribed to asphyxia when no asphyxial insult has occured.

Antepartum asphyxia is particularly difficult to diagnose and can occur when no risk factors are recognised. The brain damage which occurs following antepartum asphyxia may be a cause of intrapartum foetal distress which further confuses the diagnosis (Naeye *et al*, 1989). The use of MRI in plotting the location of neuropathological injury can be helpful in diagnosing the timing of the insult since as the foetus matures there is a differing pattern of vulnerablity across the developing brain (Barkovich and Truwit, 1990.

The overall incidence of perinatal asphyxia has been recorded between 4.8 and 11.6 per 1000 live born births per year (Brown *et al*, 1974; MacDonald *et al*, 1980 Nelson and Ellenberg, 1981; Ergander, 1983; Levene, 1983; Palme and Ericsson, 1986;). In most of the later studies a general trend towards a reduction in the incidence of perinatal hypoxic-ischaemia has been identified. A recent report observed the incidence of hypoxic-ischaemia encephalopathy between 1976-1980 and 1984-1988, in the central UK region, and identified a significant reduction in the occurrence of the insult from 7.7 per 1000 live births to 4.6 per 1000 live births (Hull and Dodd, 1992). Despite the falling incidence of hypoxic-ischaemic encephalopathy the clinical problem is still of great concern particularly in view of the fact that a greater proportion of those infants affected in recent times exhibit the moderate or severe encephalopathy (Hull and Dodd, 1992).

1.2.3 The Effects of Hypoxic-Ischaemic Insults on Non-CNS Regions

It is important to note that the brain does not suffer in isolation, and in fact is remarkably well preserved at the expense of other organs in the body. During a period of asphyxia the reduced blood oxygen levels cause a redirection of blood flow to the most essential organs, the heart, the brain and the adrenals (Stahlman, 1977; Guyton, 1991; Jensen and Berger, 1991). A great deal of the current knowledge on this circulatory centralisation has come from studies on the chronically instrumented foetal sheep (Rudolph, 1984; 1992; Jensen and Berger, 1991). In

the foetal animal the umbilical vein joins the foetal circulation in the liver and during normoxia a little over half of the highly oxygenated foetal blood is directed into the inferior vena cava by way of the ductus venosus. Preferential streaming of this blood from the right atrium to the left atrium in the heart, through the foramen ovale, ensures that the oxygen-rich supply reaches the upper body first (Jensen and Berger, 1991).

During asphyxia the ductus venosus widens to re-route a greater proportion of the umbilical venous supply directly to the upper body (Rudolph, 1984; Jensen and Berger, 1991). When oxygen delivery is compromised there is a redistribution of blood flow to maintain oxygen supply to the heart, brain and adrenal glands in the majority of experimental paradigms. Peripheral chemoreceptor activation leads to activation of parasympathetic and sympathetic pathways with a corresponding release of catecholeamines from the adrenal medulla and sympathetic nerves (Jensen, 1992). The resultant peripheral vasoconstriction reduces blood flow to the carcass, skin and scalp. Pulmonary perfusion is reduced when blood is shifted from the right side of the heart directly into the left atrium and ductus arteriosus. However, with maturation of the foetal circulation, there is an increase in direct ventricular output to the lungs so pulmonary hypoperfusion is less likely to occur. Asphyxia also results in the redirection of aortic flow into the placenta away from the kidneys, the gut and the limbs. Renal dysfunction can result in acute tubular necrosis and oliguria and the effects in the gut may induce stress ulceration and necrotizing enterocolitis (Levene, 1987; Jensen and Berger, 1991).

Prolonged asphyxia may eventually lead to cardiovascular problems. Cardiac glycogen stores protect cardiac function for prolonged periods but eventual depletion results in dilatation and poor myocardial contractile force (Stahlman, 1977; Myers, 1977). Cardiac arrest and myocardial ischaemia may occur but hypotension predominates and usually persists after the asphyxia thereby exacerbating any damage to the infant. The distress induced by the asphyxia will often cause passage of meconium and gasping can also occur which results in harmful inspiration of irritant meconium into the lungs. The presence of meconium in the lungs can be a useful indication that intrauterine asphyxia has occurred (Levene, 1987).

The metabolic dysfunction which occurs after asphyxial damage such as hypernatraemia, hypoglycaemia, hyperglycaemia and metabolic acidosis is also an indication of the duration of the asphyxia and the extent of the damage (Volpe, 1977). The clinical care of any neonatal infant suffering the effects of an hypoxic-ischaemic episode must take into account all physiological variables and not just the neurological signs. Indeed, birth asphyxia which is severe enough to damage the brain is likely to cause damage to the kidneys, lungs and even the heart (Naeye *et al*, 1989). Diagnosis and treatment of hypoxic-ischaemic insults is thus complex and time consuming if all possible areas of damage are to be considered.

1.2.4 The Neuropathology Associated with Perinatal Hypoxic-Ischaemic Brain Injury

The type of neuropathological lesions which result from an hypoxic-ischaemic episode depend largely upon whether hypoxia or ischaemia predominates (Larroche, 1985; Brierley and Graham, 1985). Focal lesions may be attributed to a purely ischaemic insult, such as neonatal stroke, but the additional complication of hypoxia can exacerbate the extent of the ischaemic lesion. Following a prolonged or severe hypoxic-ischaemic episode there may be myocardial damage and the resultant hypotension will reduce cerebral perfusion and thereby add to the primary insult (Levene, 1988; Myers, 1977). Prematurity is of major consideration in the assessment of neuropathological lesions since the maturity of the brain and the cerebral vasculature is directly related to the pattern of damage associated with hypoxic-ischaemia (Banker and Larroche, 1962; Hill and Volpe, 1981).

An infant brain removed several days after a severe hypoxic-ischaemic insult is likely to be swollen with flattened gyri and narrowed sulci. The swelling can result in a slit-like compression of the lateral ventricles and the cortex often shows grey-brown discoloration. Subdural bleeding is common and intraventricular and/or subependymal haemorrhage frequently accompanies a severe hypoxic-ischaemic insult, particularly in the premature infant (Larroche, 1985; Hill and Volpe, 1981; Levene, 1987; DeVries *et al*, 1988;Volpe, 1992).

In the following section the main patterns of brain injury following perinatal hypoxic-ischaemia will be discussed but it must be noted that each pattern may, or may not, occur in isolation and that the age of the foetus or neonate will also determine which pattern of injury predominates.

1.2.4a Periventricular Leukomalacia (PVL) and White Matter Damage

PVL is primarily a lesion of the white matter and is predominantly found in premature infants (Larroche, 1985). In an early pathological study Banker and Larroche (1962) observed PVL in 19% of all brain post-mortems performed on neonates who died at less than one month of age but in those neonates born prematurely the incidence of PVL rose to 64%. The common feature in all cases was that anoxia had been diagnosed at birth. The recent advances in risk management of obstetrical care during labour and delivery have led to increasing numbers of very low birth weight infants who survive (Volpe, 1992). The incidence of PVL in these premature infants vary between 25 and 40%.

PVL is typically found 3-10mm from the walls of the lateral ventricles and in three main regions of the brain; the first is anterior to the anterior horn of the lateral ventricles, the second area involves the corona radiata near the body of the lateral ventricles and the third area encompasses the region adjacent, and lateral, to the temporal and occipital horns of the lateral ventricles (Banker and Larroche, 1962). The first and third areas are most commonly affected and are affected to the greatest extent (Larroche, 1985). The PVL lesion consists of small white spots, which are areas of coagulation necrosis, surrounded by a chalky white ring and in the case of older lesions there may be cavitation, softening and liquefaction of the affected region (Larroche, 1985). In the most extensive lesions there is dilatation of the lateral ventricles and also intraventricular haemorrhage is frequently observed but there is little, if any, involvement of the grey matter (Larroche, 1985; Hill and Volpe, 1981; Volpe, 1977).

The cause of this type of cerebral injury has been linked to a border zone theory due to the close proximity to the terminal zones of the major penetrating cerebral arteries (Banker and Larroche, 1962). The penetrating branches of the anterior and middle cerebral and the choroidal arteries form anastomoses 3-10mm from the ventricular wall. In the immature brain the

branches are poorly developed and so hypoperfusion in these regions is believed to be the cause of PVL (DeReuck *et al*, 1972; Takashima and Tanaka, 1978). A rich leptomeningeal arterial supply over the cortex ensures that cortical regions are spared from damage in the premature brain. As the brain matures the ventriculopetal and ventriculofugal branches of these periventricular domains proliferate and anastomoses improve such that these regions are no longer vulnerable but the regression of the leptomeningeal supply exposes the cortical region to the effects of hypotension and ischaemia (Levene, 1987; Larroche, 1985). There is experimental evidence to support the border zone theory of periventricular damage due to inadequate perfusion (Abramowicz, 1964; Young *et al*, 1982) but some researchers have argued that the theory cannot account for generalised reductions in CBF and regions distant from the border zones which suffer hypoxic-ischaemic damage (Myers, 1977). The immaturity of the brain development is also critical since the actively differentiating and/or myelinating glial cells located in the periventricular zone will be particularly vulnerable to reductions in cerebral perfusion pressure (Volpe, 1992).

Clinical diagnosis of white matter damage can be difficult unless intra-ventricular haemorrhage is also a feature of the cerebral response where clinical signs such as coma, decerebrate posturing and generalised tonic seizures suggest a poor prognosis (Volpe, 1977; Hill and Volpe, 1981). Non-invasive methods of diagnosis such as real-time ultrasound are, however, available to detect the presence of PVL and the progression of the lesion can be followed when ultrasound is combined with nuclear magnetic resonance imaging (MRI) techniques (Fawer *et al*, 1985; Dubowitz *et al*, 1985; Wyatt *et al*, 1989; Barkovich and Truwit, 1990).

The main neurological correlate of this injury is spastic diplegia because the descending pyramidal tracts from the motor cortex pass through the affected area. The leg distribution is closer to the angle of the lateral ventricles and therefore the lower limbs are usually most affected. The intellect can be spared following PVL but damage to the optic radiations may cause visual defects (Pape and Wigglesworth, 1979; Larroche, 1985). The prognosis is usually good if the lesions are small (less than 3mm in diameter), confined to narrow areas and non-

cavitated but with multiple, extensive lesions accompained by cerebral haemarrhage cerebral palsy is likely to develop, with or without visual and auditory defects according to the extent of the lesion (Pape and Wigglesworth, 1979; Levene, 1987; DeVries *et al*, 1988).

The process of maturation of the cerebral perfusion occurs gradually over a considerable period of time, and because the vascular supply to the cortical and sub-cortical regions extends deeper into the cerebrum, the pattern of vulnerability to hypoperfusion changes. In the full-term infant and during the first few months of post-natal life triangular regions at the depths of the sulci are particularly sensitive to hypoperfusion because of inadequate perfusion, and the resultant sub-cortical leukomalcia is located some distance from the periventricular regions (Takashima *et al*, 1978; DeVries *et al*, 1988). The pattern of damage associated with sub-cortical leukomalacia is often much greater and more persistent than that found with PVL and as a result the prognosis is worse. In addition to the grey matter damage which accompanies this lesion the resultant neurological correlate is typically a weakness of the upper limbs (DeVries *et al*, 1988; Levene, 1987).

1.2.4b Parasagittal Neuronal Necrosis

Parasagittal neuronal necrosis also develops as a feature of hypotensive events which result in inadequate cerebral perfusion of the border zones of the major cerebral arteries (Larroche, 1985; Levene, 1987). This pattern of neuropathological injury is also referred to as 'watershed infarcts' on the premise that those regions most distant from the main source of irrigation are the first to suffer when the supply is reduced (Volpe, 1976; Volpe, 1987). The injury may predominate in one hemisphere but typically both hemispheres are equally affected with posterior regions more severely affected than anterior regions (Volpe, 1987). Parasaggittal injury usually accompanies subcortical leukomalacia as a feature of a hypotensive insult (Hill and Volpe, 1981; De Vries, 1988).

Positron emission tomography (PET) has been used to observe reductions in cerebral blood flow (CBF) in the parasaggittal regions following an episode of asphyxia. Through the use of technetium brain scans the region of reduced CBF was found to correlate with areas of tissue injury in the neonatal brain (Volpe *et al*, 1985; Volpe and Pasternak, 1977). The regions

affected involve the border zone territories between the anterior and middle cerebral arteries and those between the middle and posterior cerebral arteries (Volpe, 1976; Volpe *et al*, 1985; Larroche, 1985). Within 48 hours of the insult the affected regions may not appear to be grossly affected but microscopic investigation can identify pyknotic nuclei surrounded by shrunken cytoplasm and with time the injury may form multiple cystic lesions (Larroche, 1985).

The evidence of reduced CBF in the damaged parasaggittal areas links the cause of the injury with systemic hypotension. Hypotension may be a feature of reduced cardiovascular function following an asphyxial episode or may be caused by localised obstructions of CBF related to post-hypoxic-ischaemic cerebral oedema (Myers, 1977; Hill and Volpe, 1981; Volpe, 1987). The neurological outcome of parasaggittal cerebral injury is not entirely understood but a weakness of the upper limbs reflects involvement of the motor cortex (Volpe, 1976; Volpe, 1987). In more severe insults, where neonatal seizures occur, involvement of regions such as the caudate nucleus, globus pallidus and ventral pons and also of the cerebellar Purkinje cell layer can produce ulegyria (gyral scarring) and more extensive neurological deficits (Volpe, 1987; DeVries *et al*, 1988; Levene, 1987).

1.2.4c Selective Neuronal Necrosis

Hypoxic-ischaemic episodes are associated with extensive cellular metabolic changes and, hence, the normal metabolic requirements of a cell will determine the extent to which the hypoxic-ischaemia can damage that cell (Siesjö, 1978; Vannuci, 1990). In the developing brain the metabolic requirements of cellular regions reflect the changing pattern of differentiation and growth such that vulnerability lies at the core of the cerebrum in the premature infant. In contast in the full term infant the rapidly expanding cortical regions and laminar formation are vulnerable to injury (Towbin, 1970). Selective neuronal necrosis is a feature of hypoxic-ischaemic injury which reflects the extent to which oxygen deprivation has compromised the perinatal brain and the distribution of neuronal necrosis is directly related not only to the severity of the insult but also to the type of insult (Hill and Volpe, 1981; Levene, 1987). Experimental

evidence has shown that the occurence of several very short episodes of hypoxic-ischaemia is associated with striatal damage whereas single, acute episodes are associated with damage to the hippocampus and the cortex (Lin *et al*, 1992).

A mild insult is characterised by injury to the hippocampal pyramidal neurons and the cerebellar Purkinje cells (Volpe, 1976). Following a severe insult, however, the necrosis is often diffuse affecting not only the regions already mentioned but also cortical layers 3 and 5 particularly in the visual cortex, the large neurons of the globus pallidus, the subiculum, the thalamus and the internal granule layer and dentate nuclei of the cerebellum (Hill and Volpe, 1981; Larroche, 1985; Levene, 1988). The necrotic lesions are typically bilateral although one hemisphere may be more affected than the other (Volpe, 1976). The temporary vascular watershed at the depths of the sulci means that neurons in these regions are also vulnerable to hypoxic-ischaemic injury (Levene, 1987).

Light microscopic evaluation of the brain after a hypoxic-ischaemic insult reveals pyknotic nuclei, cytoplasmic vacuolation and neurons which are spindle-shaped (Larroche, 1985). After some months the more severe insults often produce ulegyria which is characterised as pale areas at the depths of the sulci caused by vacuolation and chromatolysis which greatly reduces the number of neurons (Hill and Volpe, 1981; Levene, 1988). The presence of multiple regions of necrosis across the cortex causes a number of shrunken scars to form giving the granular appearance referred to as 'granular atrophy' (Larroche, 1985).

The neuropathological injury following perinatal asphyxia can extend to involve the brain stem nuclei and in some cases brain stem nuclei may be selectively involved (Norman, 1972; Leech and Alvord, 1977; Hill and Volpe, 1981; Larroche, 1985). The inferior colliculus and reticular formation are especially vulnerable to injury but pontine and cranial nuclei can also be affected. The brain stem lesions are characterised by patchy irregular neuronal loss, which leads to discoloration, and can extend to involve the thalamic and hypothalamic nuclei (Norman, 1972). The pattern of brain stem involvement may be a direct reflection of the type of injury because acute, total asphyxia can produce selective brain stem and thalamic injury whereas a prolonged episode of partial asphyxia followed by secondary hypotension involves the cortex

more than the brain stem (Leech and Alvord, 1977; Myers, 1977). Where infants are known to have suffered cardiac arrest MRI scans have shown that neuropathological injury is restricted to the brain stem, thalamus and basal ganglia (Barkovich and Truwit, 1990).

Following a severe hypoxic-ischaemic, or asphyxial, episode swelling of the perivascular astrocytes, in response to ionic imbalances, and "bleb" formation from endothelial cells that project into the capillary lumen, can severely restrict vascular supply (Volpe, 1976; Levene, 1988). This "no-reflow" phenomenon causes secondary hypoperfusion and the resultant regional alterations in CBF can be detected by technetium brain scans (Ames *et al*, 1968; Levene, 1987). Clinical diagnosis is more usually achieved through neurological grading of the post-hypoxic-ischaemic encephalopathy (Volpe, 1977; Finer *et al*, 1983; Levene, 1987).

The neurological outcome associated with selective neuronal necrosis depends to a great extent upon which neurons are involved but with moderate or severe encephalopathy some measure of disability is likely (Levene, 1986). Neonatal seizures of the subtle, generalised tonic, multifocal clonic, focal clonic or myoclonic type may develop in which case the prognosis is likely to be poor (Hill and Volpe, 1981; Finer *et al*, 1983; Minchom *et al*, 1987). Long-term neurological deficits include dystonia, spastic paraplegia, spastic quadriplegia, medullary deficits, cortical blindness and variable levels of mental retardation (Volpe, 1976; Larroche, 1985; Levene, 1988).

1.2.4d Status Marmoratus

Status marmoratus selectively involves the basal ganglia and, unlike selective neuronal necrosis, is only found as a result of perinatal or neonatal and not adult hypoxic-ischaemia. The lesion is rare and takes some months to develop because it affects the formation of myelin in the basal ganglia which does not commence until around 6 months of age (Hill and Volpe, 1981; Larroche, 1985; Levene, 1988).

Status marmoratus affects the striatum, in particular the head of the caudate nuclei and small cells of the putamen, and it also involves the thalamus (Volpe, 1976). Striatal lesions are predominant in the full-term but thalamic lesions predominate in the premature infant. The substantia nigra, amygdala and mammillary bodies are rarely affected (Volpe, 1976; Larroche, 1955).

When fully developed status marmoratus creates a marbled appearance which is bilateral and is composed of spotty or irregular whitish areas and streaks in the basal ganglia (Larroche, 1985). Although focal neuronal loss and gliosis do occur, status marmoratus is due to an abnormal pattern of myelination. Instead of the usual tight axonal bundles, astrocytes form a thin network that in combination with dense patches of myelin produces a marbling effect (Larroche, 1985). When it does occur this type of neuropathology is usually associated with the extensive cerebral injury observed following perinatal asphyxia. Choreoathetosis is believed to be the neurological deficit which results from status marmoratus but additional retardation of motor and intellectual development may also be a feature of this neuropathology (Volpe, 1976; Hill and Volpe, 1981; Levene, 1988).

1.2.4e Focal Ischaemic Injury or Neonatal Stroke

As in the adult, an episode of cerebral vascular occlusion in the first few days of postnatal life can produce focal damage that if serious can infarct (Larroche, 1985; DeVries *et al*, 1988). The neonatal stroke may be caused by embolic, thrombic or ischaemic occlusion and an arterial site is more common than venous infarction (Barmada *et al*, 1979; DeVries *et al*, 1988). Ischaemic injury in the premature brain is characterised by multiple small infarcts whereas in the full-term brain there is usually a singular, large infarct (Barmada *et al*, 1979). The extent of the focal ischaemic damage depends on which vessels are occluded and the damage is frequently unilateral.

Infarcted regions are produced when all cells (neuronal, glial and vascular) are killed and this results in severe disorganisation of both grey and white matter (Hill and Volpe, 1981; Larroche, 1985). The initial oedematous swelling may distort the surrounding structures and the lateral ventricles are frequently dilated and focal ischaemic infarcts are commonly associated with haemorrhage into the lesion site (Barmada *et al*, 1979; Hill and Volpe, 1981).

The use of modern imaging techniques is of major importance in the diagnosis of neonatal stroke and using such methods an incidence of 1 in 5000 full-term deliveries was recently reported (Levene, 1987). The neurological deficits associated with neonatal stroke are varied ranging from transient neonatal seizures and childhood epilepsy to spastic hemiparesis and there may also be a small degree of mental retardation (Hill and Volpe, 1981; DeVries *et al*, 1988). In general the prognosis is quite good but it has been suggested that neonatal, ie postpartum stroke may be responsible for around 20% of cases of cerebral palsy (Levene, 1987).

1.2.5 Perinatal Hypoxic-Ischaemia, or Asphyxia, as a Cause of Long-term Neurological Illness

The neuropathological disturbances described in the previous section might suggest that any infant suffering a hypoxic-ischaemic event during the perinatal period would be likely to exhibit serious neurological deficits but this does not happen (Scott, 1976; Thomson *et al*, 1977). The severity of perinatal asphyxia varies greatly as does the physiological and pathological response of the neonate. Antenatal and postnatal care and monitoring of the human infant has improved considerably in recent years and coincidentally there has been a decline in the incidence of severe handicap (Hagberg *et al*, 1975; Levene, 1986; Hull and Dodd, 1992).

The accurate diagnosis of birth asphyxia is a particular problem in the clinic and symptoms normally associated with birth asphyxia can appear in a range of perinatal disorders (see section 1.2). Where birth asphyxia is confirmed the insult carries a very high risk and in one study it was found that 43% of stillborn infants and 47% of those who died in the first week of life had been exposed to birth asphyxia (Naeye *et al*, 1989). However, the frequency of birth asphyxia remains low and the incidence of mortality as a consequence of perinatal asphyxia has

been declining for some years but at all times the majority of infants that die took more than 20 minutes to develop spontaneous respiration or exhibited a grade III hypoxic-ischaemic encephalopathy (Wegman, 1984; Ergander *et al*, 1983; Hull and Dodd, 1992).

It has been observed that where birth asphyxia is severe enough to cause brain damage the infant is likely to die before or very soon after delivery and as a result of this between 75 and 90% of surviving infants suffer no serious handicap (Scott, 1976; Thomson *et al*, 1977; Blair and Stanely, 1988; Naeye *et al*, 1989; Nelson and Leviton, 1991). In a comprehensive survey of 43,437 full-term infants only 6% of the cases of cerebral palsy were attributed to birth asphyxia (Naeye *et al*, 1989). However, of the 9 chidren who suffered cerebral palsy as a result of birth asphyxia a greater proprtion suffered from the more serious quadriplegic disability where all four limbs were affected. The greatest number of cases of cerebral palsy were due to congenital CNS malformations the early symptoms of which can easily be mistaken for birth asphyxia which suggests that over the years reports on the incidence of serious handicap resulting from birth asphyxia might have been misleading (Nelson and Leviton, 1991). In addition the low frequency of cases of cerebral palsy in full-term infants attributable to birth asphyxia suggests that in the majority of cases of cerebral palsy the brain damage occured some time before labour and delivery (Bedrick, 1989).

The technical advances in obstetrical care and paediatric management have resulted in ever increasing numbers of surviving premature infants. However, the immaturity of the CNS vasculature and germinal matrix as well as the immaturity of protective autonomic nervous system responses means that birth asphyxia can have very serious consequences (Volpe, 1992; Jensen and Berger, 1991). Between 5 and 15 % of surviving very low birth weight infants suffer from cerebral palsy and an additional 25-50 % are likely to suffer learning disabilities (Volpe, 1992).

The occurrence of intraventricular haemorrhage with periventricular leukomalacia and the early development of neonatal seizures are two features which typically jeopardise the infant's chance of survival (Levene, 1983; Fawer *et al*, 1985; Minchom *et al*, 1987). Levene (1986) estimated that around 600 infants in the United Kingdom would die or suffer serious

handicap every year as a result of perinatal hypoxic-ischaemia. In a recent study the incidence of severe hypoxic-ischaemic encephalopathy was found to be unchanged despite a fall in the overall incidence of perinatal hypoxic-ischaemia (Hull and Dodds, 1992).

There is also the possibility that the cerebral metabolic and vascular disturbances created during perinatal hypoxic-ischaemia may cause a minor handicap without evidence of gross neuropathological damage (Towbin, 1971). Minimal brain dysfunction is characterised by hyperactivity and motor disturbances accompanied by learning disorders and one possible cause of this cerebral disorder is mild hypoxic-ischaemia particularly in the prenatal phase.

Perinatal hypoxic-ischaemia is of considerable interest to investigators not least because of the potential therapeutic benefit of improved diagnostic and interventional methods. The differing patterns of pathological and neurological injury which result from episodes of perinatal hypoxic-ischaemia reflect not only the different duration and severity of the insult but also the constantly evolving pattern of vulnerability as the CNS develops and matures.

1.3 Animal Models of Hypoxic-Ischaemic Brain Injury

Systemic hypoxia leads to reduced arterial oxygen concentration (hypoxemia) and thereby reduces the vital supply of oxygen to tissues. The systemic response to hypoxia involves a sympathetic autonomic response of baroreceptors in the carotid bodies thereby causing an increase in the arterial blood pressure. Simultaneous activation of peripheral chemoreceptors causes peripheral vasoconstriction thereby adding to the circulatory centralisation in favour of the heart and brain in an attempt to compensate for the lack of tissue oxygen (Rudloph, 1984; Guyton, 1991). However, if the oxygen deficit is prolonged the energy deprived myocardial muscle beats slower and less efficiently which results in systemic hypotension and thereafter reduced cerebral blood flow (CBF). If hypoxemia is associated with increased arterial carbon dioxide (hypercapnia) and increased plasma acidity, this can be described as asphyxia (Siesjö, 1978; Siesjö, 1981; Plum, 1983; Myers, 1977).

A wide range of animal models of hypoxic-ischemic brain injury have been developed to reproduce the pathophysiology seen in human anoxia, hypoxia, ischaemia and asphyxia in attempts to understand further the neuropathological profile, the neurological outcome and the appropriate therapeutic intervention required to prevent damage. In this section I will discuss the adult animal models of hypoxic-ischemia, and thereafter will discuss in more detail perinatal animal models of hypoxic-ischaemic brain injury.

1.3.1 Adult Models of Hypoxic-Ischaemia

Human cerebral hypoxic-ischaemic injury is caused either by a global insult, such as cardiac arrest, carbon-monoxide poisoning or near-drowning, or by a focal insult, such as a stroke or haemorrhage (Brierley and Graham, 1985; Choi, 1990). The two types of injury are differentiated by the regional distribution of the reduction in CBF and experimenters have tried to reproduce the injury patterns with a variety of animal models. It is important to differentiate between different models of hypoxic-ischaemia because a small residual blood flow during or after the insult; e.g., with incomplete global ischaemia, often causes more severe damage than total cessation of CBF (Rehnorona *et al*, 1981; Plum, 1983; Choi, 1990).

1.3.1a Global and Forebrain Ischaemia

The neuropathological damage following a brief episode of global hypoxic-ischaemia is restricted to selectively vulnerable regions of the brain. These regions include layers 3, 5 and 6 of the neocortex, subfields of the hippocampus (in particular the CA1 field) and the subiculum. In more severe models of global ischaemia the dorsolateral portions of the caudate and putamen, certain thalamic nuclei and the Purkinje cells of the cerebellum are also vulnerable to injury (Brierley and Graham, 1985). Cellular necrosis follows a distinct temporal profile referred to as ischaemic cell change which begins with microvacuolation of the cell, then incrustation of the plasma membrane may occur which is followed by cell death (Brierley and Graham, 1985; Kirino, 1982).

The reduction of CBF to less than 5% of control levels can be achieved through several procedures including raised cerebrospinal fluid pressure, a neck tourniquet, and in the most severe case decapitation (see Ginsberg and Busto, 1989; Neely and Youmans, 1963; Nordstrom *et al*, 1978; Duffy *et al*, 1972). The majority of these studies, however, are concerned with the metabolic consequences of global ischaemia. The most-widely used models of global ischaemia are the 2-vessel occlusion plus hypotension and the 4-vessel occlusion models (Pulsinelli and Brierley, 1979; Smith *et al*, 1984a). The 2-vessel occlusion model involves bilateral clamping of the common carotid arteries and exsanguinous lowering of the systemic blood pressure to 50mmHg (Smith *et al*, 1984a). CBF is reduced to 5% of control levels in the neocortex and to 15% of control levels in the hippocampus and striatum (Blomquist *et al*, 1984). Limited brain damage has been associated with an occlusion of just 2 minutes but the more reproducible 10-30 minute occlusion results in necrosis of the CA1 and CA4 hippocampal pyramidal cells, the subiculum, the neocortex and striatum. The worst damage is found over the superlateral portion of the hemisphere and in the middle layers of the neocortex.

In the 4-vessel occlusion model the vertebral arteries are occluded through the use of electrocoagulation and during this surgical procedure clips are placed around the common carotid arteries. 24 hours later the carotid clamps are tightened for a maximum of 30 minutes to

create a 95% reduction in forebrain regional CBF (Pulsinelli and Brierley, 1979; Pulsinelli *et al*, 1982a). In both models the reduced cerebral blood flow is followed initially by a period of hyperaemia upon release of the clamps and then by a long period of hypoperfusion (6-24 hours).

If the period of ischaemia is brief the depression of the rodent's conscious state that occurred early in the hypoxic-ischaemia is rapidly overcome upon reperfusion with no apparent neurological consequences. The neuropathological damage is restricted to the CA1 region of the hippocampus, layers 3, 5 and 6 of the neocortex, in particular the posterior cortex, and the striatum, but there is no infarction or haemorrhage. This rodent model tends to be highly strain specific and attempts to replicate the results with different strains of rats have produced wide variations in the reduction of CBF (Blomquist *et al*, 1984; Ginsberg and Busto, 1989).

The pathological changes associated with global ischaemia follow a distinct temporal profile, referred to as delayed neuronal death, such that the small striatal neurons exhibit ischaemic cell changes soon after the insult but damage of the larger CA1 pyramidal cells is delayed by 24-72 hours (Kirino, 1982; Pulsinelli *et al*, 1982b).

Less complete reductions in CBF are found with the Levine model of unilateral carotid occlusion (Levine, 1960). This model combines the unilateral carotid artery occlusion with a gradual anoxia in a nitrogen-enriched atmosphere. In the modified method, the combination of hypoxia and ischaemia produces cellular necrosis in the ipsilateral hippocampus, cortex, striatum, thalamus and midbrain (Levy *et al*, 1975). The unilateral hypoxic-ischaemia also causes white matter damage and post-insult increases in CBF result in oligaemia in the ipsilateral hemisphere (Salford *et al*, 1973).

1.3.1b Focal Ischaemia

The majority of animal investigations into focal ischaemia have involved electrocoagulation of the middle cerebral artery (MCA) via the temporal approach described by Tamura and colleagues (1981a). Recently several new models have been described as alternatives for the induction of focal ischaemia, including the rose bengal photosensitising dye which in concert with light irridiation causes localised platelet aggregation (Watson *et al*, 1985; Ginsberg and Busto, 1989). The lesion produced in the MCA occlusion model is greatly

dependent upon the exact portion of the vessel which has been occluded such that the extent of the neuropathological damage corresponds with the area of reduced regional CBF (Tamura *et al*, 1981b; Chen *et al*, 1986). The reduction of CBF to 12-15ml/100g/minute consistently produces histological damage (Naritomi *et al*, 1988; Shimada *et al*, 1989) and the infarct produced in this model is often surrounded by a thin (1-2mm) transitional zone referred to as the penumbra where there is a gradual increase in the local CBF towards the outer edge of this zone (Tyson *et al*, 1984; Nedergaard, 1987, Astrup *et al*, 1981).

A focal ischaemic insult is restricted to produce an area of pannecrosis which corresponds to the area normally perfused by the occluded cerebral blood vessel. The infarct produced in a focal insult is unlike the necrosis seen in global ischaemia such that all cells, including neurons, glia, vessels and nerve fibres in the ischaemic core are victims of coagulative necrosis (Brierley and Graham, 1985). The penumbral region surrounding the densely ischaemic core exhibits selective neuronal necrosis with intact glia and vessels. The insult is frequently associated with cerebral oedema which if severe can cause herniation and a shift of the midline structures. There is no evidence of selective vulnerability in the core and the infarct is located around the middle cerebral artery territory.

The global and focal animal models of adult hypoxic-ischaemic brain damage correlate well with the neuropathological damage seen in human cerebral injury but their main advantage is as tools for a better understanding of the mechanisms of damage and for testing neuroprotective agents (Plum, 1983; Choi, 1990).

1.3.2 Animal Models of Perinatal Hypoxic-Ischaemic Brain Damage

During the first half of this century a great deal of obstetric maintenance was related to analgesia, sedation and anaesthesia (see Myers, 1977). The 'apnoea neonatorum' which was frequently seen in neonates was commonly believed to be the major cause of brain injury in the newborn infant. This association of birth apnoea with 'asphyxia neonatorum' formed the basis of the early attempts to reproduce perinatal brain damage (Windle and Becker, 1943; Ranck and Windle, 1959). These early models will be discussed in the context of the pathophysiological

effects they produced and their lack of correlation with the patterns of neuropathological damage in the human neonate. The later, more appropriate models of partial hypoxic-ischaemic damage in the perinatal animal will then be discussed.

1.3.2a Anoxia or 'Total Asphyxia Neonatorum' as a Model of Perinatal Brain Damage

In an early model Windle and colleagues (Windle and Becker, 1943; Bailey and Windle, 1959) occluded the uterine vessels supplying near-term foetal guinea-pigs until 'respiratory movements' could no longer be detected (10-19 minutes) and then delivered the guinea-pigs by Caesarian section. The neurological defects produced in these experiments were transient and variable but some lasting spasticity of the forelimbs was associated with the most severe insults, and frequently the weights of the post-asphyxial animals were lower than those of the controls animals. The neuropathological damage produced in this model was restricted to subcortical structures, particularly the brain stem and the thalamus, and that damage was minimal (Windle and Becker, 1943).

The induction of total asphyxia in foetal monkeys was achieved through detachment of the placenta and keeping the foetus in the intact amniotic sac (Ranck and Windle, 1959). After a brief (11-16 minutes) insult the monkeys were resuscitated and allowed to recover. Some of the monkeys were severely disabled but they rarely developed seizure activity and brain damage was restricted to the brain stem in particular the inferior colliculus. The maintenance of the intact amniotic sac prevented physiological monitoring of the foetus during the asphyxia and this model was adapted to an occlusion of the umbilical cord (Windle, 1966; Cooke *et al*, 1958; Myers, 1972). In the newly delivered monkey foetus the umbilical cord was clamped and a saline-filled rubber sac was placed over the monkey's head to prevent air breathing. The occlusion of the umbilical cord halted the fetal blood circulation although a transient increase in arterial blood pressure was detected by some investigators (Myers, 1972). During the occlusion there was a gradual decline in cardiac output as the heart rate slowed and eventually, after a brief secondary rise, the arterial blood pressure fell to that of the surrounding tissue (2-3mmHg).

Resuscitation could be achieved after a 35 minute period of asphyxia but if the asphyxia lasted for more than 20 minutes permanent myocardial damage later led to the onset of cardiogenic shock and the death of the neonatal monkey (Myers, 1972).

Windle (1966) and Myers (1977) reported that an insult of just 8-12 minutes resulted in transient neurological impairment and that asphyxia lasting longer than 15 minutes caused long-term disability. The newly resuscitated monkeys were clumsy and hyperactive with postural abnormalities and a lasting spasticity of the extremities and Windle (1966) argued that these neurological defects mimicked those seen in human cerebral palsy.

The minimum duration of asphyxia required to cause irreversible neuronal necrosis was 14 minutes (Myers, 1977) and a distinct pattern of increasing vulnerability was seen as the severity of the asphyxia increased. The areas affected were predominantly brain stem nuclei starting in the inferior colliculus and extending into the superior olivary nucleus, the descending nucleus of the 5th cranial nerve, the lateral and spinal vestibular nuclei and the gracile and cuneate nuclei. Thalamic damage occurred in the posterior and ventral nuclei and following the more severe insults the Purkinje cells of the cerebellum were destroyed. Haemorrhage or infarction was rare and there was no cortical damage or oedema.

The occurrence of brain stem damage has been found in the human neonate (Norman, 1972; Leech and Alvord, 1977) but it is rare to see post hypoxic-ischaemic human perinatal brains without cortical damage. The neurological defects associated with this model may include spasticity but reproducibility was poor and post-asphyxial seizures common in the human did not develop.

1.3.2b Hypoxic-Ischaemia (Partial Asphyxia) as a Model of Perinatal Brain Damage

The threat of hypoxic-ischaemic brain injury in the human foetus is often linked to maternal circulatory problems such as placenta previa or disorders of uterine mobility (see section 1.2, and Myers, 1977). Animal models frequently reflect this hazardous association between mother and child through the induction of foetal hypoxic-ischaemic insults, e.g. via compression of the maternal abdominal aorta (Myers, 1972), halothane induced maternal hypotension (Brann and Myers, 1975), reduced oxygen-carrying capacity in the maternal blood

through carbon monoxide inspiration (Ginsberg and Myers, 1976), reduced oxygen concentration in the maternal inspired air (Mann, 1970; Myers, 1977), intravenous infusion of oxytoxic agents and infusion of catecholamines into the mother (see Myers, 1977).

In a hypoxic environment the foetal monkey arterial partial pressure of oxygen had to be reduced by 90% for a period of longer than 25 minutes if brain damage was to occur, and such an insult caused severe depression in heart rate and blood pressure that was often not recoverable (Myers, 1977). Systemic hypotension, caused by a prolonged oxygen deficit, reduced cerebral blood flow leading to hypoxic-ischaemic changes in cortical areas but preservation of flow to the brain stem ensured a lack of damage in this area (Myers, 1977; Vannucci and Duffy, 1977).

Following delivery after partial asphyxia the resuscitated animals exhibited visible abnormalities in the upper limbs but the most severely affected monkeys developed a decerebrate posture and, within 4-12 hours also developed tonic-clonic seizures. In the surviving animals seizures did not develop and within 24 hours the neurological signs had improved significantly (Myers, 1977).

The brains of the animals which were killed, or died, within a few hours of delivery exhibited widespread oedema, flattening of the convolutions and softening of the cerebral cortex. There was extensive cortical necrosis, particularly of the outer layers, in the paracentral and posterior parietal regions. Hippocampal and striatal damage was also found although the dentate gyrus was rarely damaged. The most severe insult was usually accompanied by infarcted regions of the neocortex which, in premature animals, was frequently accompanied by haemorrhage. Deep subcortical structures were well preserved and brain stem damage was rare. Microscopic evaluation of necrotic regions highlighted a massive increase in intracellular volume at the expense of the extracellular space and the subcellular mitochondria were swollen and had fragmented membranes. Post hypoxic-ischaemic oedema caused brain swelling raising the intra-cranial pressure and the resulting constriction of cerebral vessels was thought to exacerbate damage (Myers, 1977).

Although white matter damage was not found in the full-term monkey foetus, prenatal exposure to hypoxia during gestation did later result in white matter necrosis in the fetal sheep. Damage to the basal ganglia was only found if the hypoxic insult was later followed by a post-natal anoxic episode such as is caused by respiratory distress syndrome (Myers, 1977).

The profile of hemispheric damage found after partial asphyxia correlates well with patterns of damage seen in the human infant brain following hypoxic-ischaemia (Hill and Volpe, 1981; Larroche, 1985). Those animals which exhibited extensive cortical necrosis, with or without haemorrhage, rarely survived beyond 48 hours which is similar to the group III hypoxic-ischaemic encephalopothy found in the clinic (Fenichel, 1983; Levine, 1987). The animals which recover normal neurological function within 24 hours showed only minimal cortical damage which may be a suitable model of minimal brain dysfunction (Towbin, 1971).

The acutely exteriorised fetal sheep has also been used as a model for total asphyxia, via the umbilical cord clamp, and partial asphyxia, via clamping of the maternal aorta (Rosen *et al*, 1976; Kjellmer *et al*, 1974; Rosen *et al*, 1986). In the model of partial asphyxia the cardiovascular responses induced by occlusion of the maternal aorta have been shown to preserve CBF and cerebral metabolic derangement was only associated with the more severe, prolonged insults (Rosen *et al*, 1986; Hagberg *et al*, 1987a).

The chronically instrumented foetal sheep preparation has been of considerable value in the understanding of foetal physiological responses to a range of hypoxic insults (Rudolph, 1984; Jensen and Berger, 1991). It is possible to investigate mechanisms in the unanaesthetised foetus using this model, and through the use of radiolabelled microspheres a detailed knowledge of blood flow changes throughout the foetal body during hypoxia has been gained. The chronic preparation has been applied to 'total' asphyxia via reduction of umbilical blood flow and to 'partial' asphyxia via maternal hypoxemia, graded reduction of uterine blood flow and arrest of uterine blood flow (see Rudolph, 1984 and Jensen and Berger, 1991). Each insult is associated with some degree of foetal bradycardia and raised foetal arterial blood pressure as well as compensatory redistribution of blood flow to protect the essential organs.

Maternal hypoxemia is associated with a reduction in foetal arterial pO_2 from 24 mm Hg to 10-12 mm Hg and the severity of this reduction in oxygen delivery produces marked changes in blood flow distribution whereby the flow to the heart, brain and adrenals is increased and that to the lungs, kidneys, gut and carcass is decreased. A similar centralisation of foetal blood flow is seen with a graded reduction in uterine blood flow but following arrest of uterine blood flow, cerebral blood flow does not change and instead the cerebral vascular resistance alters to increase flow to the brain stem at the expense of the cerebrum and the choroid plexus (Jensen *et al*, 1991; Jensen, 1992). It has been shown that blood flow changes are influenced by the oxygen content of the blood and to reduce total body oxygen consumption oxygen delivery must be reduced by 50% (Jensen *et al*, 1991; Field *et al*, 1990). For cerebral oxidative metabolism to be compromised the oxygen saturation of circulating cerebral blood must be lower than 1.0 mM at which point the compensatory mechanisms cannot be extended to maintain sufficient oxygen delivery (Field *et al*, 1990).

The foetal guinea-pig has also been used in a model of acute asphyxia induced by arrest of uterine blood flow (Berger *et al*, 1991; Jensen, 1992). In this paradigm the mother is decapitated and as the first foetus is delivered by Caesarean section the uterine artery is clamped. A second foetus is delivered after 2 minutes of asphyxia and a third is delivered after 4 minutes of asphyxia (Berger *et al*, 1991). This model conformed to the view that oxygen delivery to the brain is reduced even during brief episodes of acute asphyxia and anaerobic metabolism caused a decrease in brain tissue glucose and an increase in lactate. However, when the insult remains brief compensatory increases in cerebral blood flow during a recovery period can prevent neuronal damage (Jensen, 1991).

Two models of prenatal asphyxia in the near-term foetal rat have recently been described. In the first model, acute uteroplacental occlusion of one uterine horn produced biochemical changes associated with hypoxic-ischaemic neuronal damage but no neurological or pathological observations were made (Magal *et al*, 1988). In the second model asphyxia was induced by 'delayed Caesarian section' where the entire uterus was removed and placed in a $37^{o}C$ water bath for up to 17 minutes (Bjelke *et al*, 1991). The foetal heart rate declined gradually through the duration of the asphyxia but oxygen saturation measurements were

inconclusive. During the early neonatal period the rearing behaviour of the hypoxic rats was reduced and their weight gain was also less compared with age-matched controls. The neuropathological changes included a reduction in cell density in the CA1 and CA3 regions of the hippocampus and also a proliferation of dopaminergic neurons in the substantia nigra. It remains to be proved whether or not these models produce long-lasting physiological changes compatible with total or partial asphyxia and how useful they will be in modelling human perinatal asphyxia.

1.3.3 Neonatal Hypoxic-Ischaemia

The seven day old neonatal rat is thought to be at an equivalent stage of cerebral development to the full-term human foetus (Rice *et al*, 1981). The Levine (1960) model of unilateral carotid occlusion has been combined with a prolonged hypoxia (8% oxygen) for 2-3.5 hours to create a unilateral cortical infarct in the 7 day old rat (Rice *et al*, 1981; Andine *et al*, 1990). Dwyer (1988) has shown that the hypoxic insult must commence within 4 hours after the surgical ligation to prevent compensatory mechanisms from the contralateral hemisphere reducing the infarct size. The recovery from the insult is rapid and 79% of surviving animals show no visible neurologic abnormalities (Vannucci, 1990).

In rats exposed to the hypoxic-ischaemia neuronal damage was found in the ipsilateral cortex, striatum and hippocampus with an accompanying swelling of the affected hemisphere (Rice *et al*, 1981; Andine *et al*, 1990). Cortical necrosis was organised in columnar patterns at right angles to the pial surface and the most severe necrosis was found in the posterior cortex. The CA1 region of the hippocampus was particularly vulnerable but the CA3-CA4 regions also suffered moderate damage. Damage in the striatum and thalamus tended to be moderate. White matter necrosis did occur in regions of myelinogenesis especially along the dorsolateral portion of the callosal radiation (Rice *et al*, 1981).

The temporal profile of the damage in this model is faster than in the adult models of global hypoxic-ischaemia such that even 15 hours after the insult the microvacuolation typical of early ischaemic cell change was not visible. The oligomesodermal reaction, which is proportional to the time after the insult in adult rats, was proportional to the extent of the damage in the neonatal rat (Rice *et al*, 1981).

Ikonomidou and colleagues (1989a) have modified the model of hypoxic-ischaemia by replacing the hypoxia with 75 minutes in a hypobaric atmosphere. The most extensive lesions produced with these conditions were found in 6 day old rats and the distribution of neuronal necrosis was similar to that observed in the hypoxic-ischaemia model (Ikonomidou *et al*, 1989b; Rice *et al*, 1981). The 10 day old rat, however, was used for a full evaluation of this model and although no explanation was offered it would appear that the distribution of damage was more reproducible and the mortality rates lower when 10 day old rats were used (Ikonomidou *et al*, 1989a,b). Interestingly, 125 minutes in the hypobaric chamber were required to produce any brain damage in the 2 day old rat but even this length of insult could not produce the same extent of damage as found in the older rats (Ikonomidou, 1989b).

A model of asphyxia in neonatal piglets has proved extremely valuable in the study of regional alterations in cerebral blood flow (CBF) during and after the insult (Goplerud *et al*, 1989). In one study aspyhxia was induced by cessation of mechanical ventilation in anesthetised, paralysed 3-8 day old piglets. Total blood flow to the brain during aspyhxia did not increase but there was a regional alteration of the CBF in favour of the brain stem away from the cerebral gray and white matter and the choroid plexus. However, during recovery there was a uniform increase in blood flow to all regions of the brain and the relative homogeneity of CBF distribution was restored. Similar changes in CBF distribution have been demonstrated in the brains of asphyxiated newborn dogs (Vannucci and Duffy, 1977, Koons *et al*, 1993). Such models are of interest in the study of mechanisms responsible for blood flow changes during asphyxia, particularly with respect to the differnces between perinatal and adult adaptive responses.

These neonatal models are used extensively in the study of perinatal hypoxic-ischaemic brain damage particularly in the evaluation of therapeutic intervention (Vannucci, 1990). Few studies have investigated long-term recovery or the neurological consequences of these models and their correlation with human perinatal hypoxic-ischaemic brain injury is yet to be proved.

1.4 The Tolerance of the Perinatal Animal to Oxygen Deprivation

The extent to which the brain has developed by the time of birth varies a great deal amongst species and it may, or may not, be related to the gross physical development of the species. In general the mammals can be divided into two groups at birth, the precocial, or relatively mature, animals such as the cow, horse, sheep and guinea-pig, and the non-precocial, relatively immature, animals such as the dog, cat, rat, mouse and the human (Himwich, 1962). In evolutionary terms the precocial and non-precocial division would appear to have arisen from the amount of neonatal care provided by the parent and from the measure of independence required for the neonate to survive. The extent to which a young animal is expected to fend for itself also greatly determines the rate at which post-natal maturation of the CNS proceeds (Himwich, 1951; McIlwain and Bachelard, 1985).

There are many features of metabolic and neurophysiological development which characterise the late foetal and early neonatal life of all species but these are beyond the scope of this thesis (for reviews see Jones and Rolph, 1985; Himwich, 1962; Himwich, 1976; McIlwain and Bachelard, 1985). The pace and extent at which cerebral metabolic alterations occur corresponds to the patterns of changes involved in cerebral maturation such as cell growth, process formation, myelination, and, in particular, the development of electrical activity. Overall the metabolic alterations reflect the developing functional capacities of the different cerebral regions (McIlwain and Bachelard, 1985).

There is one particular feature of cerebral maturation which is of considerable interest to this study and that is the remarkable tolerance exhibited by foetal and neonatal animals to oxygen deprivation (Fazekas *et al*, 1941; Stafford and Weatherall, 1960; Duffy *et al*, 1975). Foetal tolerance to anoxia, a condition that can be produced by placing an animal in a nitrogen (N_2) atmosphere, is even greater than that of the neonate (Dawes *et al*, 1959; Duffy *et al*, 1975). A number of cardiovascular and cerebral adaptive responses are believed to be related to this perinatal tolerance to asphyxia. The oxygen-carrying capacity of foetal blood is already at a considerable advantage over the adult because foetal haemoglobin has a higher affinity for oxygen than adult haemoglobin. Furthermore, in certain species including humans the blood concentration of foetal haemoglobin is significantly greater than that in maternal blood which

further enhances the oxygen carrying capacity of foetal blood (Rudolph, 1992). Oxygen delivery to the foetal organs is directly related to blood flow which means that any changes in foetal blood flow will directly affect the oxygen supply to the foetal organs.

Studies which have employed the use of the chronically instrumented foetal sheep have demonstrated that the enhanced ability of the foetus to withstand a reduced oxygen supply is associated with a circulatory and metabolic centralisation in favour of the heart, brain and adrenals (Rudolph, 1984; 1992; Jensen and Berger, 1991; Jensen, 1992). In experiments where oxygen supply has been limited through maternal hypoxemia or graded reduction of uterine blood flow, the nature of the circulatory response is such that foetal blood flow to the peripheral organs, in particular the lungs, carcass, skin and scalp, is reduced. In contast, a rise in foetal arterial blood pressure promotes an increase in blood flow to the heart, brain and adrenals. The enhanced preferential streaming of oxygen-rich umbilical venous blood through the ductus venosus and the foramen ovale ensures that oxygen delivery to the heart and brain is maintained. As blood flow to the peripheral organs is reduced so too is oxygen delivery, and these studies demonstrate that oxygen consumption in the foetus is related to oxygen delivery in a linear fashion (Jensen and Berger, 1991; Jensen, 1992). A rise in fractional oxygen extraction across the arterio-venous vascular bed of peripheral organs can initially protect oxygen consumption. However, as oxygen delivery continues to fall alterations in oxygen extraction can no longer compensate and total body oxygen consumption falls. However, the redistribution of blood flow to the heart and brain maintains oxygen delivery and therefore oxygen consumption. In other words, the circulatory centralisation is accompanied by a centralisation of oxidative metabolism (Jensen and Berger, 1991).

The increase in myocardial and cerebral blood flow is believed to be mediated by local vascular effects triggered by a reduced oxygen content of the circulating blood (Rudolph, 1984). The reduction in pulmonary circulation is also believed to be caused by a locally mediated vasodilation. However, it has been demonstrated that stimulation of the autonomic nervous system occurs concurrently with the peripheral vascular responses (Jensen and Berger, 1991; Jensen, 1992). A reduction in foetal arterial pO_2 stimulates peripheral arterial chemoreceptors, eg in the carotid sinus, which causes an increase in sympathetic tone thereby producing a rise in

plasma levels of noradrenaline and adrenaline which are released from sympatheic nerves and the adrenal medulla. Foetal arterial blood pressure rises and peripheral vasoconstriction reduces blood flow to the peripheral organs. The response of the arterial chemoreceptors can be potentiated by a rise in arterial pCO_2 and a fall in blood pH. Reduced oxygen consumption in the periphery promotes anaerobic metabolism resulting in metabolic acidosis which can stimulate arterial chemoreceptors and further reduce oxygen delivery to the peripheral organs (Jensen, 1992).

In support of the role of the sypathetic nervous system, it has been shown that blockade of the sympathetic reponse by ß-adrenoceptor antagonists exaggerates the fall in foetal heart rate, and blood flow to the heart, brain and adrenals is not maintained (Jensen and Berger, 1991). Ablation of the sympathetic nervous system by chemical sympathectomy also alters the initial cardiovascular reponse and blood flow is increased to the carcass at the expense of the heart and spinal medulla (Jensen and Lang, 1991). The stimulation of the sypathoneuronal and sympathomedullary systems by reduced foetal arterial pO_2 is associated with a rapid rise in plasma catecholeamines which occurs in concert with circulatory redistribution via peripheral vasoconstriction, which in turn reduces oxygen delivery to, and therefore oxygen consumption by, the peripheral organs. Total body oxygen consumption falls but this allows preferential delivery of oxygen to the heart, brain and adrenals where blood flow is increased and hence oxygen consumption is maintained. Should the asphyxia be severe and prolonged then the circulatory centralisation cannot be maintained and severe metabolic disruption and acidemia ($pH < 7.00$) can result in serious damage to the foetus (Jensen, 1992).

The resistance of the cardiovascular system, which is also related to high levels of carbohydrate stores, is a major component of the perinatal anoxic tolerance (Dawes *et al*, 1959; Stafford and Weatherall, 1960). However, certain features of the immature nervous system are also believed to play a significant part in the prolonged survival of the perinatal animal during an episode of oxygen deprivation (Duffy and Vannucci, 1977). In an anoxic environment an adult rat exhibits a period of excitement then enters a comatose state before finally succumbing to anoxia (Fazekas *et al*, 1941). In the study of Fazekas and colleagues (1941), it was observed that not all perinatal animals exhibit the same level of anoxic tolerance such that the neonatal rat

survived an average 50 minutes in 100% N_2 whereas the relatively mature neonatal guinea-pig survived only 7 minutes of anoxia. The foetal rat succumbs to a 100% N_2 atmosphere with an LD_{85} of 45 minutes (which is the time by which 85% of the foetal rats have died) that is 5 times longer than the 7 day old rat and almost twice as long as the one day old rat (Vannucci and Duffy, 1976; Duffy *et al*, 1975). The fact that the heart was still beating, even though the respiratory effort had ceased, further supported the role of the nervous system in limiting anoxic survival.

The circulatory centralisation demonstrated in the foetal sheep shows that oxygen delivery to the foetal brain is maintained allowing cerebral oxidative metabolism to be maintained (Rudolph, 1984; Jensen, 1992). Maternal hypoxemia is associated with a reduction in cerebral vascular resistance and a failure of autoregulation so that the cerebral blood flow (CBF) is pressure passive and changes in relation to raised foetal arterial blood pressure. These adaptive mechanisms mean that the foetal sheep brain can maintain a constant cerebral oxygen consumption over a wide range of arterial pO_2 from 14-36 mm Hg (Field *et al*, 1990). In situations where cerebral oxygen delivery falls the raised cerebral oxygen extraction can maintain some level of oxygen consumption. However, if oxygen delivery continues to fall beyond maximal oxygen extraction then cerebral oxidative metabolism may fail (Jensen and Berger, 1991).

Experiments which have studied acute and chronic hypoxemia demonstrate a heterogeneity in the raised CBF with the greatest increase to the medulla, followed by the subcortex, pons and cerebellum and the smallest increase in CBF is to the cortex (Richardson *et al*, 1993). In the case of acute asphyxia induced by the arrest of uterine blood flow, total blood flow to the brain does not increase but there is an increase in cerebral vascular resistance so that flow to the brain stem increases at the expense of flow to the cerebrum and choroid plexus (Jensen and Berger, 1991; Berger *et al*, 1991). This redistribution of CBF protects the vital autonomic nerve centres located in the brain stem and appears to be mediated by raised levels of catecholeamines due to sympathetic activation (Jensen, 1992). The reduction in oxygen delivery to the cortex, subcortex and cerebellum during acute asphyxia is associated with reduced oxygen consumption and, as a result of anaerobic metabolism, raised levels of lactate (Berger *et al*, 1991). Even brief episodes of acute asphyxia can take cerebral oxygen delivery to

critically low levels but 2-3 fold increases in CBF during recovery are more than adequate to compensate for reduced oxygen consumption during the brief insult (Jensen, 1992).

The near-term foetus and neonate are typically able to utilise anaerobic mechanisms for the derivation of cerebral metabolic energy and the pentose-phosphate pathway is of particular importance to the perinatal animal for the provision of substrates for lipid and protein deposition during growth of the cerebrum (Takagaki *et al*, 1974; McIlwain and Bachelard, 1985). The enzymes necessary for oxidative metabolism, eg succinate dehydrogenase, are present only in low quantities at birth in animals such as the rat, cat, dog and human; however, these enzymes mature rapidly during the early postnatal phase as the animal becomes more dependent upon aerobic respiration (Himwich, 1976; McIlwain and Bachelard, 1985). The activity of the enzyme, pyruvate dehydrogenase matures particularly slowly and it is this which is thought to critically determine the switch from anaerobic metabolism to dependence upon aerobic metabolism (Cremer, 1982; McIlwain and Bachelard, 1985).

The lower cerebral metabolic energy requirement and poor synaptic organisation of the perinatal animal are thought to be major components of the anoxic tolerance phenomenon (Thurston and McDougal, 1969; Duffy *et al* 1975; Aghajanian and Bloom, 1967). The observation that sodium iodoacetate, which inhibits glycolysis, greatly reduces survival is supportive of the critical role of anaerobic glycolysis in anoxic tolerance (Samson and Dahl, 1957). The foetal rat is known to exist in a state of hypercapnia with a carbon dioxide tension (pCO_2) almost twice that of the neonate (Vannucci and Duffy, 1974). During an anoxic episode foetal CO_2 tension rises more rapidly, and to a greater degree, than in the neonate. Extreme hypercapnia is thought to reduce the cerebral metabolic rate and this may prolong anoxic survival of the foetus (Vannuci and Duffy, 1976).

The total amount of the high energy phosphate compounds and precursors declines during anoxia but to a greater degree in neonatal rats than in foetal rats (Vannucci and Duffy, 1976). In both age groups, however, cerebral energy expenditure during anoxia is considerably less than that found in the adult. The level of foetal rat brain phosphocreatine (PCr) is around 45 times less than that found in the one day old rat and ATP-ase activity in the perinatal animal is also very low (Vannucci and Duffy, 1974; Himwich, 1962; McIlwain and Bachelard, 1985).

These factors are important when considering the low level of electrophysiological activity in the perinatal rat brain.

The rodent EEG matures postnatally between 10-20 days of age which corresponds with growth of dendritic processes, cortical synapse formation, myelination, increases in neurotransmitter concentrations and the enzymes of transmitter metabolism, such as glutamic acid decarboxylase and acetylcholinesterase, and the observation of K^+ and Na^+ ion homeostasis via the ATP-ase ion pump (McIlwain and Bachelard, 1985; Aghajanian and Bloom, 1967; Altman, 1972; Grove *et al*, 1973). As the rodent brain continues to mature the level of high energy phosphate reserves rise correspondingly and by pnd 14-18 the duration of anoxic survival is similar to that in the adult (Tofts and Wray, 1985; Fazekas *et al*, 1941).

The resting cerebral energy consumption of a one day old rat has been estimated to be around 5% of the adult and the neonate survives 25 times longer than the adult rat in an anoxic environment (Duffy *et al*, 1975). Similarly the resting cerebral energy consumption of a 7 day old rat is estimated to be around 10% of an adult and the infant rat survives 9 times longer in 100% N_2 than the adult (Duffy *et al*, 1975). Moreover, the regional cortical glucose utilisaton (rCGU) in the 7 day old rat is between 70 and 92% lower than that found in corresponding regions of the adult rat brain (Vannucci *et al*, 1989). Alterations in cerebral vascular resistance cause a redistribution of CBF in favour of the brain stem where critical respiratory control centres are located in the neonate as well as a foetal animal (Vannucci and Duffy, 1977; Goplerud *et al*, 1989). This redistribution, in addition to the reduced cerebral metabolic requirements of the neonatal and 7 day old rat, appears to be related to the duration of survival in a nitrogen atmosphere.

It has been demonstrated that lactate can be utilised as an alternative substrate to glucose, and is used in preference to glucose for cerebral metabolism in the foetal rat such that lactate can provide more than 70% of the cerebral metabolic energy requirement (Dombrowski *et al*, 1989). Other species such as the dog and the sheep are also thought to be able to utilise lactate, and lactate dehydrogenase activity is frequently high in neonates (Cremer, 1982; Thiringer *et al*, 1982). The uptake of lactate from blood to brain is considerably greater in the perinatal animal than the adult and is believed to be mediated by a stereospecific carrier as well as by non-

saturable diffusion across a blood brain barrier more permeable to lactate (Cremer, 1982; Vannucci and Mujsce, 1992). In contrast, the uptake of glucose in the one day old rat is around 20% of the adult rate and around 33% of the adult rate in the 7 day old rat (Moore *et al*, 1971; Cremer *et al*, 1976).

The content of lactate in foetal brain tissue and blood is greater than in the neonatal rat but during anoxia lactate levels rise in a linear fashion in both age groups (Vannucci and Duffy, 1974; 1976). The rise in brain tissue lactate is not simply a result of enhanced anaerobic glycolytic flux but also because of accelerated uptake of lactate from blood to brain by the carrier-mediated mechanism. A feature of the early recovery phase following anoxia is the continued uptake of lactate from blood to brain and the coversion of lactate to pyruvate which can be further oxidised in the tricarboxylic acid cycle (Duffy *et al*, 1975; Vannucci and Duffy, 1976). The use of lactate in preference to glucosè means that brain tissue glucose tends to accumulate above control levels in the early recovery phase (Vannucci and Duffy, 1976).

Ketone bodies, in particular ß-hydroxybutyrate and acetoacetate, form another class of alternative substrates for oxidative metabolism in the suckling rat (Hawkins *et al*, 1971; Dombrowski *et al*, 1989). The uptake of ketone bodies from blood to brain is believed to be mediated by the same carrier mechanism that is used for lactate and pyruvate uptake in the perinatal rat brain (Cremer, 1982). During recovery from anoxia, when oxygen becomes available, the neonatal rat is able to utilise ketone bodies in the same way that the foetal rat oxidises lactate thereby accelerating the rate of recovery over that observed in the adult rat. The use of ketone bodies as alternative substrates is not universal for all species but human infants are believed to be able to utilise ß-hydroxybutyrate and acetoacetate in times of extreme stress such as starvation (Himwich, 1976).

Circulatory and metabolic centralisation as well as reduced rates of glucose metabolism and the lower metabolic requirements of the perinatal brain are undoubtedly the major attributes that govern the anoxic tolerance phenomenon but additional features might also be considered. The non-precocial animal is also essentially poikilothemic when born, with a large surface area to volume ratio, which is of particular relevance to the observation that a lower environmental temperature extends the neonatal survival period in 100% N_2 (Fazekas *et al*, 1941; Stafford and

Weatherall, 1960). The lack of temperature regulation may be yet another feature which contributes to the extraordinary anoxic tolerance of the immature offspring of many mammalian species.

1.5 Mechanisms of Hypoxic-Ischaemic Cell Damage

The pathological and neurological outcomes of all forms of hypoxic-ischaemic brain insults are directly related to a complex array of vascular and cellular disturbances which include metabolic alterations, ionic fluxes, neurotransmitter release and loss of intracellular calcium homeostasis (see Siesjö, 1981; Siesjö, 1984; Choi, 1990; Vannucci, 1990; Kjellmer, 1991). The mechanism by which hypoxic-ischaemic cell death occurs is not yet fully understood but what is known is that the final destiny of the necrotic cell is dependent upon more than one process of destruction. Focal and global hypoxic-ischaemic episodes lead to an extensive range of metabolic and neurochemical tissue alterations but in the case of global ischaemia it is the events in the reoxygenation and recirculation period that are believed to critically determine the fate of the affected region of the brain. The putative mechanisms of hypoxic-ischaemic cell damage will be discussed with particular reference to events in the perinatal brain.

Adult global ischaemia causes a generalised reduction in cerebral blood flow (CBF), the extent of which is dependent upon the method of induction of the ischaemia. In the immediate recovery phase after a moderate global ischaemic insult there is a reactive hyperaemia, and this is followed by a prolonged phase of secondary hypoperfusion (Pulsinelli *et al*, 1982b; Hossman, 1982; Kagstrom *et al*, 1983a and b). The metabolic rate increases very slowly during recovery such that during early recovery oxygen supplies exceed demand (hyperoxia). However, as the metabolic rate continues to increase and the CBF falls, a secondary phase of oxygen deprivation can occur (see Siesjö, 1981). Recovery can be limited with only minimal restoration of CBF in certain areas and persistent metabolic deficits if the ischaemic insult is severe or prolonged (Welsh *et al*, 1978; Welsh *et al*, 1982a).

1.5.1 Metabolic Alterations During Hypoxic-Ischaemia

The balance between energy supply and demand critically determines the efficient functioning of the brain and under normal conditions that balance is expressed in the difference between ATP utilisation, during cellular metabolic work, and rephosphorylation of ADP to regenerate ATP supplies. The rate of consumption of ATP and production of ADP directly

influences the rate of oxidative phosphorylation in the mitochondrial electron transport system which provides the energy to regenerate ATP. In the adult glucose is the usual substrate for oxidative metabolism and a continued supply of blood glucose and oxygen is necessary for the continued support of the energy balance (see Siesjö, 1978; and Vannucci, 1990).

Hypoxia causes a reduction in arterial oxygen tension but in an attempt to maintain an adequate supply of oxygen CBF is increased and if hypoxia occurs in isolation metabolic changes are limited to an elevation in brain tissue lactate and a reduction in PCr concentrations (Duffy *et al*, 1972; Siesjö, 1978). Incomplete global ischaemia combined with graded hypoxia worsens the metabolic deficit with an additional disruption in ATP phosphorylation but glucose supply is maintained (Salford and Siesjö, 1974).

Following the onset of global ischaemia in the adult the cellular energy balance is upset and high energy phosphate compounds are used up. Brain tissue PCr declines to zero levels within 1 minute of the onset of ischaemia whereas ATP levels decline rapidly between 5-7 minutes after the onset on ischaemia (Duffy *et al*, 1972; see Siesjö, 1981). The transitory preservation of ATP levels is related to the alternative mechanisms of ADP rephosphorylation via creatine kinase and adenylate kinase but ATP levels are ultimately limited by the reduced level of PCr as well as uncoupling of oxidative phosphorylation (Siesjö, 1978).

The energy crisis caused by the oxygen deficit results in reduced ATP and increased levels of ADP and AMP which stimulate phosphofructokinase, thereby promoting acceleration of the glycolytic flux (Siesjö, 1978). In the absence of oxygen anaerobic glycolysis predominates and as the oxidation of NADH fails the increased levels of NADH stimulate the lactate dehydrogenase (LDH) equilibrium to produce lactate from pyruvate (Duffy *et al*, 1972; Siesjö, 1984). The production of ATP and lactate from anaerobic glycolysis is small and is limited by preischaemic stores of glucose and, in the foetal animal, glycogen.

The onset of global ischaemia is associated with, in sequence, reduced PCr, increased lactate, reduced ATP, increased ADP and AMP and decreased brain tissue glucose and glycogen with maximal alterations occurring within 10 minutes of the onset of ischaemia (Siesjö, 1984). If adequate perfusion pressure is restored recovery of metabolites following a moderate global ischaemic insult is usually rapid and complete (Siesjö, 1981). The conversion of AMP to

adenosine following ischaemia reduces the adenine nucleotide pool and this, accompanied by loss of the nucleotides and bases during recirculation, can delay the postischaemic recovery of ATP levels (Siesjö, 1984).

1.5.2 The Role of Lactacidosis in Hypoxic-Ischaemia

Considerable attention has been focussed on the elevation of brain tissue lactate during ischaemic insults and the possible role of lactacidosis in ischaemic injury. During complete global ischaemia, where glucose supply is limited, minimal elevation of lactate is followed by complete recovery but during incomplete global ischaemia a residual supply of glucose allows excessive lactate formation to over 25 nmols/mg tissue and metabolic recovery is poor (Nordstrom *et al*, 1976).

The nutritional state of the ischaemic subject is critical because administration of glucose to monkeys prior to cardiac arrest reduced functional recovery and augmented neuropathological injury (Myers and Yamaguchi, 1977). In a later more comprehensive study, preischaemic administration of glucose was associated both with two-fold increases in lactate compared with increased lactate levels in fasted ischaemic animals, and also with more extensive neuronal injury (Rehncrona *et al*, 1981; Kalimo *et al*, 1981). The investigation of lactate elevation demonstrates two important features of ischaemia; the first is that the outcome after incomplete global ischaemia may be worse than that after complete cessation of CBF in fed animals (Hossman and Kleihues, 1973), and the second is that hyperglycemia exacerbates ischaemic neuronal damage (Kalimo *et al*, 1981). The common feature in both cases is an excessive elevation of brain tissue lactate to between 16-25nmols/mg tissue after which metabolic recovery is highly unlikely (Rehncrona *et al*, 1981; Plum, 1983; Siesjö, 1984). Injection of lactic acid into the cerebral cortex of adult rats also produces histological damage similar to that associated with hypoxic-ischaemic injury (see Vannucci and Mujsce, 1992). The term lactacidosis must, however, be used with caution since lactate accumulation is not always directly linked with pH reduction (Paschen, 1987).

1.5.3 Metabolic Alterations During Perinatal Hypoxic-Ischaemia

The perinatal animal displays a remarkable tolerance to anoxia and hypoxia and an hypoxic-ischaemic insult must be severe or prolonged if irreversible necrosis is to occur (Section 1.4; Fazekas *et al*, 1941; Duffy *et al*, 1975; Vannucci, 1990). Metabolic alterations during hypoxic-ischaemia in the perinatal animal are similar to those in the adult particularly with respect to the decrease in high energy phosphates and the increase in lactate. In the 7 day old rat model of hypoxic-ischaemia (Rice *et al*, 1981), PCr levels declined to 14% of control and ATP levels declined to 26% of control levels (Palmer *et al*, 1990). There was also a delay in ATP regeneration during recovery from hypoxic-ischaemia in the perinatal rat such that the adenine pool only reached 81% of the control level when all other metabolites had recovered (Palmer *et al*, 1990).

Lactate accumulation has been shown to occur in foetal and neonatal animals during hypoxic-ischaemia as a result of enhanced anaerobic glycolytic flux (Duffy *et al*, 1975; Chao *et al*, 1989; Palmer *et al*, 1990). An *in utero* hypoxic-ischaemic insult in the near-term foetal rat led to an elevation of brain tissue lactate to 29nmols/mg (Magal *et al*, 1988), and in the 7 day old rat model of unilateral hypoxic-ischaemia (Rice *et al*, 1981), lactate rose to over 10nmols/mg in both the ischaemic and non-ischaemic brain hemispheres (Palmer *et al*, 1990). The accumulation of lactate in both hemispheres where only one is destined to be damaged suggests that acidosis associated with lactate does not cause hypoxic-ischaemic brain damage in the immature brain (Vannucci and Mujsce, 1992). However, brain damage in the 7 day old rat does correlate with pH reductions associated with NADH accumultion in the ischaemic hemisphere (Welsh *et al*, 1982b). In addition, as lactate forms an alternative energy substrate in the perinatal brain, it is unlikely that lactate accumulation contributes in any way to perinatal hypoxic-ischaemic brain injury (Palmer *et al*, 1990; Vannucci, 1990).

Hyperglycemia, which is harmful to the adult, has been shown to increase survival times during perinatal hypoxic-ischaemia (Stafford and Weatherall, 1960; Voorhies *et al*, 1986; Vannucci and Mujsce, 1992), and does not exacerbate hypoxic-ischaemic brain damage (Dawes *et al*, 1964; Miller, 1971; Voorhies *et al*, 1986). Glucose uptake in the 7 day old rat brain is around one fifth of the adult rate. Although cerebral uptake is accelerated during hypoxic-

ischaemia, the low activity of hexokinase prevents over-activation of glycolysis such that glucose utilisation rates are comparable for normoglycemic and hyperglycemic infant rats (Duffy *et al*, 1975; Vannucci, 1990; Vannucci and Mujsce, 1992). This means that overproduction of lactate does not occur in the 7-day old rat model and lactate accumulation is in fact comparable in both hemispheres in the unilateral hypoxic-ischaemic model (Palmer *et al*, 1990). Glucose supplementation may therefore be of value in the protection of pregnancies or neonates where asphyxial risk factors have been identified.

Hypoxic-ischaemia in both adult and perinatal animals is associated with considerable energy derangement and accumulation of brain tissue lactate. The metabolic deficits following moderate ischaemia are readily reversed although there is a delay in the complete regeneration of the high energy phosphate ATP. These changes must now be considered in the context of other cellular events during hypoxic-ischaemia.

1.5.4 Ionic Fluxes During Hypoxic-Ischaemia in the CNS

Following the onset of global ischaemia the EEG flattens within 10-20 seconds and evoked responses are abolished within 4 minutes (Hansen, 1985). The EEG remains isoelectric throughout the duration of the insult and for some time during recovery, depending on the extent of the insult. Following recovery neuronal activity returns and can continue to increase to values above those recorded in control situations although some investigators have found a persistent depression of spontaneous neuronal activity for up to 24 hours of recovery (Andine *et al*, 1991a; Imon *et al*, 1991).

Cellular ion homeostasis in cerebral tissues is dependent upon membrane gradients, the majority of which are maintained by ATP dependent mechanisms such that 50-60% of cellular energy expenditure is used to maintain ionic gradients (Erecinska and Silver, 1989). The Na^+/K^+ exchange and Na^+/Ca^{2+} antiporter are two important gradients controlled by ATP hydrolysis in the neuronal plasma membrane .

The decrease in ATP, which results from the uncoupled oxidative phosphorylation, during hypoxic-ischaemia leads to a failure of the Na^+/K^+ ATPase thereby causing an increase in the extracellular K^+ concentration. ATP decline may also trigger K^+ conductances across

the neuronal plasma membrane (Hansen, 1985). The hypoxic-ischaemia induced rise in extracellular K^+ occurs in three distinct phases, an initial slow rise, a second rapid flux and a final slow rise (Hansen, 1985; Siesjö, 1984; Siesjö, 1990). Hypoxic-ischaemia is also associated with an influx of Na^+ and Cl^- ions as well as osmotically obligated water. The rise in intracellular lactate concentrations also promotes osmotic influx of water so that overall the extracellular space is reduced.

The initial slow leak of K^+ into the extracellular space is probably due in part to the reduced levels of ATP. Additionally it is likely that a membrane K^+ conductance is triggered either by the rise in free intracellular calcium or by a fall in ATP (Siesjö, 1990). When extracellular K^+ concentrations reach 10-15μM, the resultant presynaptic depolarisation induces Ca^{2+} influx which causes transmitter release leading to the stimulation of transmitter receptors (e.g. excitatory glutamate receptors) thereby opening further ion channels (Watkins and Olverman, 1987; Collingridge and Lester, 1989). The opening of agonist operated ion channels promotes a rapid flux of K^+ into the extracellular space as Na^+ and Cl^- enter the cells. The resultant depolarisation of postsynaptic elements allows the depolarising Mg^{2+} block of the NMDA-type of excitatory amino acid receptor to be relieved (Nowak et al, 1984; Collingridge and Lester, 1989) which opens a channel allowing further Na^+/K^+ conductances as well as a Ca^{2+} influx. In addition to agonist operated calcium channels, depolarisation also opens voltage sensitive calcium channels, such as the N and L-type, which promotes a rapid rise in intracellular calcium (see Siesjö and Bengtsson, 1989 and Siesjö, 1990). The "shock" opening of a multitude of ionic conductances overwhelms the compromised ATP levels, and ion pumps cannot be maintained such that ionic gradients cannot be restored. The lack of ATP and disruption of ionic homeostasis critically affects the internal cellular calcium homeostasis which leads to a release of calcium from internal stores such as the endoplasmic reticulum (see section 1.5.6).

ATP hydrolysis and NADH and lactate accumulation during hypoxic-ischaemia can all cause increases in intracellular H^+ and a concomitant decrease in intra- and extracellular pH. The collapse of the Na^+ gradient leads to a failure of the Na^+/H^+ antiporter which also results in an increase in intracellular H^+. Intracellular pH declines by 0.5 units during moderate global

ischaemia but in the centre of a focal ischaemic infarct pH can decline much further below pH 6.5 (Naritomi *et al*, 1988; Siesjö, 1984; Choi, 1990). The acid shift during hypoxic-ischaemia influences many metabolic reactions, including the LDH equilibrium, activates lysosomal enzymes which may cause autolysis, and also reduces intracellular Ca^{2+} sequestration (see Siesjö, 1978). The elevation of NADH during neonatal hypoxic-ischaemia in the 7 day old rat has been measured using fluorescent techniques and the columnar pattern of fluorescence was found to correspond with the pattern of cortical necrosis (Welsh *et al*, 1982b). The increased acidosis associated with NADH is a more likely cause of hypoxic-ischaemic neuronal death in the neonatal rat than that associated with lactate (Palmer *et al*, 1990; Vannucci, 1990). Intracellular pH has also been found to decline in foetal rat brains during exposure to an *in utero* hypoxic-ischaemic insult (O'Shaugnessy *et al*, 1991).

Glial ionic buffering is important during hypoxic-ischaemia where the rise in extracellular K^+ levels promotes glial uptake of Na^+ and Cl^- and the concomitant influx of water results in astrocytic swelling which can impede the transport of oxygen when reflow is established (Stewart and Rosenberg, 1979; Hertz, 1981; Kimelberg, 1979). Although astrocytic swelling occurs following global ischaemia there is no microscopic evidence of astrocytic membrane disruption whereas after focal ischaemia the excessive astrocytic swelling is accompanied by ruptured astrocytic membranes (Brierley and Graham, 1985). It is likely that glial ion pumping e.g. through HCO_3^-/Cl^- exchange and oxygen-dependent K^+ uptake, as well as transmitter uptake, regulates the post-ischaemic environment following global ischaemia thus preventing cytotoxic oedema and thereby aiding recovery (Plum, 1983; Siesjö, 1984; Garthwaite, 1992).

After moderate ischaemia restoration of ATP levels allows the restoration of ionic gradients which all recover to pre-ischaemic levels. However, the extracellular pH remains low for some time which may be related to the extrusion of H^+ via the Na^+/H^+ antiporter (Siesjö, 1984).

1.5.5 Neurotransmitter Changes During Hypoxic-Ischaemia

1.5.5a Glutamate and Adult Hypoxic-Ischaemia

The excitatory neurotransmitter glutamate is believed to play a significant role in the development of hypoxic-ischaemic neuronal necrosis (Meldrum, 1985; Rothman and Olney, 1986; Choi, 1990). There is a considerable body of evidence to support the role of glutamate in the induction of both adult and perinatal hypoxia-ischaemic damage (Choi, 1990; Vannucci, 1990; Kjellmar, 1992).

The retinopathy induced by systemic injection of L-glutamate in mice initiated a chain of studies which led to the suggestion that glutamate itself may be neurotoxic (Lucas and Newhouse, 1957; Olney, 1969). In cultured neurons it was observed that hypoxic damage was only found in mature cultures and that this damage could be prevented by the addition of 10mM $MgCl_2$ to the culture medium (Rothman, 1983). Furthermore anoxic neuronal death in cultures could be prevented by the addition of a glutamate antagonist, γ-D-glutamylglycine (DGG) (Rothman, 1984). In the hippocampal slice preparation, increased extracellular Mg^{2+} concentration was found to reduce the anoxic vulnerability of neurons (Kass and Lipton, 1982), and magnesium exerts a depolarising block on glutamate activated channels (Nowak *et al*, 1984). The association of the spreading depression phenomenon and glutamate/ aspartate (Van Harreveld, 1959) further promoted the link between anoxic cell death *in vitro* and glutamate excitotoxicity.

Glutamate induced neurotoxicity of cultured cortical neurons was found to be calcium dependent and cell death developed in two distinct phases (Choi, 1985). The first, reversible phase involved neuronal swelling and was dependent upon exogenous Na^+ and Cl^- ions. The second, irreversible phase produced the onset of necrosis, and this delayed phase was dependent upon the presence of extracellular Ca^{2+} (Choi, 1987; Choi, 1988). MacDermott and colleagues (1987) found that the activation of the NMDA-type of glutamate receptor led to an influx of Ca^{2+} through the agonist-operated channel.

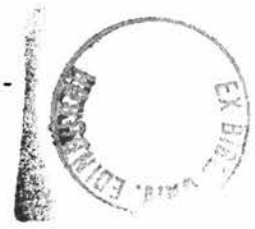
Evidence to support the role of glutamate *in vivo* was provided by the extracellular increase of glutamate and aspartate concentrations in the hippocampus during transient, forebrain ischaemia (Benveniste *et al*, 1984). A knife-cut lesion of the hippocampal CA3-

Schaffer collateral-CA1 tri-synaptic circuit also reduced ischaemic damage (Jorgensen and Diemer, 1982; Onodera *et al*, 1986) even if the CA3 lesion was performed immediately after the ischaemia (Johansen *et al*, 1987). The interruption of the CA3-Schaffer collateral input to the selectively vulnerable CA1 pyramidal neurons was found to reduce the extracellular efflux of glutamate during ischaemia as well as necrosis of the CA1 cells (Benveniste *et al*, 1989).

The induction of hypoxic-ischaemia causes an efflux of excitatory and inhibitory amino acids from intra- to extracellular regions (Hagberg *et al*, 1985; Hillered *et al*, 1989) and glutamate is elevated to levels known to be toxic in cultured neurons (Choi, 1988). The morphology of hypoxic-ischaemic neuronal necrosis is also similar to that found with glutamate excitotoxicity (Ikonomidou *et al*, 1989b).

Antagonism of glutamate receptors *in vivo* has been found to protect selectively vulnerable neurons from ischaemic damage (Simon *et al*, 1984; Gill *et al*, 1987) and it is particularly through neuroprotection that the role of glutamate has been evaluated. The excitatory action of glutamate is believed to be mediated through the action of several receptors which include the NMDA receptor, the AMPA/quisqualate receptor and the kainate (KA) receptor (Collingridge and Lester, 1989). Amelioration of hypoxic-ischaemic damage has been achieved through the use of NMDA antagonists such as MK801 and CPP-ene (Swan *et al*, 1988; Park *et al*, 1988; Bullock *et al*, 1990) and other glutamate receptor antagonists (Sheardown *et al*, 1990; Buchan *et al*, 1991). Glutamate receptor blockade would appear to be an effective way of reducing hypoxic-ischaemic neuronal damage but several paradoxes exist particularly with respect to the use of NMDA receptor antagonists in preventing global hypoxic-ischaemic damage.

Antagonism of the NMDA receptor is particularly effective in the reduction of infarct size in focal ischaemia (Albers *et al*, 1989) where the elevation of extracellular amino acids correlates well with the degree of damage (Butcher *et al*, 1990) but results in models of global ischaemia are conflicting (see Buchan, 1992). In models of global ischaemia the raised glutamate levels return to preischaemic levels early in the recovery phase (Andine *et al*, 1991a; Globus *et al*, 1991) yet post-ischaemic administration of NMDA antagonists has been found to be neuroprotective (Gill *et al*, 1988). Where the use of NMDA-receptor antagonists, such as



MK-801, has reduced global ischaemic injury (Gill *et al*, 1987) it is argued that the protective effect was due to a reduction in brain temperature (Buchan and Pullsinelli, 1990) because when normothermia was maintained MK-801 was found to have no effect on the extent of the neuronal injury. The protective influence of hypothermia has been shown in a reduction in post-ischaemic blood brain barrier disruption (Dalton-Dietrich *et al*, 1990) as well as reduced neuronal injury (Busto *et al*, 1989a; Minamisawa *et al*, 1990). The mechanism by which hypothermia protects may well be mediated through a reduction in transmitter release as well as by favourable effects on metabolic rates (Busto *et al*, 1989b; Simpson *et al*, 1991; Miller, 1971).

The conflicting reports concerning neuroprotection in global ischaemia by NMDA antagonists may be related to reduced electrical viability and lingering extracellular acidosis during early reflow as well as efficient post-ischaemic glutamate uptake (Morad *et al*, 1988; Choi, 1990; Garthwaite, 1992). However, a non-NMDA glutamate antagonist (NBQX), which acts at AMPA receptors, has been shown to reduce global ischaemic injury when administered in the early post-ischaemic period (Nellgard and Wieloch, 1992). A combination of NMDA and non-NMDA receptor activation is likely to play a role in hypoxic-ischaemic damage but the action of glutamate must be placed in context of other ongoing mechanisms of cell death during and after the insult (Choi, 1990; Albers *et al*, 1992; Buchan, 1992).

1.5.5b Other Transmitter Systems Affected in Adult Hypoxic-Ischaemia

Glutamate neurotransmission is not the only transmitter system to be affected in hypoxic-ischaemia. Striatal levels of dopamine are increased 500 fold during transient forebrain ischaemia (Globus *et al*, 1988) and unilateral lesions of the substantia nigra not only reduce the efflux of dopamine they also protect against ischaemic damage in the ipsilateral striatum (Globus *et al*, 1987). The dopamine efflux may also influence glutamate toxicity by inhibition of the glutamate uptake mechanisms (Kerkerian, 1987). Hippocampal noradrenaline levels are increased during ischaemia (Globus *et al*, 1989) and metabolic turnover of noradrenaline is also increased during ischaemia (Miyauch *et al*, 1989). The lesioning of

noradrenergic neurons in the locus coeruleus prior to forebrain ischaemia exacerbates hippocampal and cortical necrosis (Blomquist *et al*, 1985; Davies, 1987) so it may be argued that noradrenaline release during ischaemia reduces the possible extent of neuronal necrosis.

Extracellular levels of adenosine have been observed to rise during global ischaemia (Hagberg *et al*, 1987b). The adenosine A_1 receptors are implicated in hypoxic-ischaemia such that adenosine agonists (Evans *et al*, 1988; von Lubitz *et al*, 1988) and adenosine uptake blockers (De Leo *et al*, 1988; Hagberg *et al*, 1990) reduce injury but adenosine antagonists increase ischaemic brain damage (Rudolphi *et al*, 1987; Wieloch *et al*, 1985). Adenosine inhibits K^+ induced glutamate release (Dolphin and Archer, 1983) and the adenosine uptake blocker propentofylline reduces extracellular glutamate efflux during ischaemia (Hagberg *et al*, 1990) and so the protective action of adenosine may be related to effects on glutamate neurotransmission.

1.5.5c Changes in Neurotransmitters during Perinatal Hypoxic-Ischaemia

The occlusion of the umbilical cord of the exteriorised fetal lamb causes an elevation in extracellular levels of glutamate, aspartate, taurine and GABA in the cortex and basal ganglia (Hagberg *et al*, 1987a). The levels of the amino acids are lower than are found in the adult but it must be noted that uptake of glutamate is much slower than in the adult (see Kjellmer, 1991). The extracellular levels of glutamate, GABA and other amino acids are elevated even further in the 7-day old rat model of hypoxic-ischaemia (Andine *et al*, 1991b) but the pathophysiological effects of glutamate in the perinatal brain are different to those in the adult due to several developmental irregularities (McDonald and Johnston, 1990). The density of NMDA receptors increases during development to a maximal level at pnd8 which is greater than that seen in adult rats (Tremblay *et al*, 1988) and these receptors are functional such that NMDA neurotoxicity is greatly enhanced in the 7 day old rat (McDonald *et al*, 1988). The toxicity of glutamatergic compounds in the neonatal rat is reversed from the adult, and kainate exhibits no toxic action until pnd16 (McDonald *et al*, 1990; Wolff and Keilhoff, 1984). The sensitivity to hypobaric-ischaemic injury follows a similar developmental profile to the sensitivity to NMDA toxicity with high resistance on pnd2 and peak sensitivity on pnd6 (Ikonomidou *et al*, 1989b).

The transient increase in NMDA receptors is also accompanied by a transient glutamatergic innervation of regions such as the globus pallidus (Greenamyre *et al*, 1987; Barks *et al*, 1988). The potentiated response to glutamate and NMDA in the neonate is, however, reduced by NMDA antagonists (see McDonald and Johnston, 1990). NMDA antagonists also reduce the extent of neuronal necrosis and oedema following hypoxic-ischaemia in the neonatal rat (McDonald *et al*, 1987; Andine *et al*, 1988b). Post-hypoxic administration of MK-801 also reduces neonatal hypoxic-ischaemic injury (Hattori *et al*, 1989) and this may be associated with a prolonged glutamate elevation due to a transitory decrease in glutamate uptake following the insult (Silverstein *et al*, 1990). A hypersensitivity to excitatory amino acid receptor activation has not been shown in the foetus but administration of MK-801 either before or up to 6 hours after the induction of cerebral ischaemia in the foetal sheep prevented post-ischaemic seizures (Espinoza and Parer, 1991). Pretreatment with MK-801 has also been found to reduce the extent of the neuropathological damage in the foetal sheep preparation (Kjellmer, 1990).

The dopaminergic system is also affected in neonatal unilateral hypoxic-ischaemia, such that post-ischaemic levels of dopamine in the ipsilateral striatum are reduced and the characteristic asymmetric turning associated with unilateral dopamine depletion is observed (Silverstein and Johnston, 1984). Receptor studies showed a transient decrease in D_1 dopamine receptors and a more lasting decrease in D_2 dopamine receptors however uptake mechanisms were unaffected (Kostic *et al*, 1991; Przedborski *et al*, 1991). Striatal tyrosine hydroxylase immunoreactive staining was enhanced in the adult following perinatal hypoxic-ischaemia which suggested an increase in dopaminergic innervation (Burke *et al*, 1991). There was also a proliferation of tyrosine hydroxylase positive neurons in the zona compacta and pars lateralis of the substantia nigra 3 weeks after asphyxia induced at birth (Bjelke *et al*, 1991). The functional significance of such alterations in dopaminergic systems is unknown but enhanced locomotion post-asphyxia, suggests a link with hyperactivity in human minimal brain dysfunction (Bjelke *et al*, 1991).

The extensive lesion produced in unilateral hypoxic-ischaemic injury in the neonate is most likely associated with the transiently enhanced glutamate toxicity and as proof of this glutamate receptors are markedly decreased following the insult (Silverstein *et al*, 1987;

Silverstein *et al*, 1990). Other transmitter systems may be enhanced after the insult to compensate for the reduced numbers of glutamate receptors (Burke *et al*, 1991; Kostic *et al*, 1991).

1.5.6 Disruption of Calcium Homeostasis as a Potential Mechanism of Hypoxic Ischaemic Cell Death

1.5.6a Maintenance of Intracellular Calcium Concentrations

The regulation of a low intracellular Ca^{2+} concentration is vital for the protection of cellular viability because Ca^{2+} is a major regulator of intracellular metabolic reactions (Siesjö, 1981; Greenberg *et al*, 1987; Siesjö and Bengtsson, 1989; Vannucci, 1990). Free intracellular Ca^{2+} is usually held below 10^{-7}M although total intracellular Ca^{2+} is around 10^{-3}M suggesting that the cell is dependent upon a number of Ca^{2+} extrusion mechanisms, e.g. a $3Na^{+}/Ca^{2+}$ antiporter and a Ca^{2+} ATPase.

The majority of intracellular Ca^{2+} is bound to the plasma membrane and Ca^{2+} is also sequestered into the endoplasmic reticulum, and in neurons into synaptic vesicles (Borle, 1981; Siesjö and Bengtsson, 1989). Intracellular Ca^{2+} is also bound to regulatory proteins such as calmodulin and troponin as well as to Ca^{2+}-binding proteins (Choi, 1988; Siesjö and Bengtsson, 1989).

1.5.6b Intracellular Ca^{2+} Changes During and After Hypoxic-Ischaemia

As already mentioned the level of intracellular Ca^{2+} rises rapidly during hypoxic-ischaemia but falls back to control levels in the immediate recovery period (Siesjö, 1984). The accumulation of intracellular Ca^{2+} during hypoxic-ischaemia can be caused by Ca^{2+} entry via Ca^{2+} channels, by failure of Ca^{2+} extrusion mechanisms and by release of Ca^{2+} from intracellular stores.

There are several types of Ca^{2+} channels including the agonist-operated calcium channels (AOCC) and the voltage-sensitive calcium channels (VSCC). During hypoxic-ischaemia the extracellular concentrations of both excitatory and inhibitory neurotransmitters are raised (Benveniste *et al*, 1984; Hagberg *et al*, 1985) and therefore glutamate activation of the

NMDA receptor opens a Ca^{2+} conductance into the cell (MacDermott *et al*, 1986; Mayer, 1987). The continued disruption of K^+ and Na^+ conductances during hypoxic-ischaemia also results in the depolarising activation of VSCC; e.g. L-type, thereby adding to the Ca^{2+} influx (Siesjö and Bengtsson, 1989; Siesjö, 1990).

In addition to increased Ca^{2+} entry ATP depletion means that Ca^{2+}-ATPase and Na^+/K^+ATPase both fail during hypoxic-ischaemia and Na^+ influx during the insult causes a reversal of the $3Na^+/Ca^{2+}$ antiporter thereby increasing intracellular Ca^{2+}. Calcium stimulated phospholipase C activation leads to the production of inositol triphosphate (IP_3), which releases Ca^{2+} from the endoplasmic reticulum, as well as diacylglycerol which in turn activates protein kinase C leading to phosphorylation of receptor and channel proteins and further release of certain neurotransmitters including glutamate (Kjellmer, 1990; Espinoza and Parer, 1991). Intracellular metabolic and pH disruption during hypoxic-ischaemia also reduced Ca^{2+} sequestration into endoplasmic reticulum (Abercrombie and Hart, 1986) and ATP-dependent sequestration mechanisms also fail (see Siesjö and Bengtsson, 1989; Espinoza and Parer, 1991).

The establishment of reflow after global ischaemia allows oxygen to reach the energetically disabled region and therefore the cells can begin to restore normal Ca^{2+} concentrations. Initially the energy produced by the recommencement of mitochondrial electron transport is used to restore and maintain mitochondrial ion gradients and as cytosolic H^+ and Na^+ concentrations fall mitochondrial Ca^{2+} uptake greatly exceeds efflux (Siesjö and Bengtsson, 1989; Simon *et al*, 1984b). This futile cycling of ions across the mitochondrial membrane diverts the metabolic energy away from ATP production thereby compromising the restoration of the neuronal plasma membrane ion gradients as well as the restoration of ATP-dependent reactions within the cell.

Assessment of Ca^{2+} levels during and immediately after hypoxic-ischaemia has shown decreased extracellular Ca^{2+} concentrations in many brain regions (Harris *et al*, 1981; Hansen, 1985), and during the early recovery phase mitochondrial Ca^{2+} concentrations have been found to increase dramatically particularly in the CA1 region of the hippocampus (Hossman *et al*, 1985; Simon *et al*, 1984). The elevation of mitochondrial Ca^{2+} levels is interesting with

respect to the fact that mitochondrial swelling, and separation of the cristae, is one of the earliest indicators of hypoxic-ischaemic cell change (Brown and Brierley, 1968; Myers, 1972; Brierley and Graham, 1985). Ca^{2+} uptake and sequestration is severely disrupted during and immediately after a hypoxic-ischaemic insult but widespread intracellular accumulation of Ca^{2+} during hypoxic-ischaemia cannot be used as an immediate indication that cell death will occur since those cells destined to survive also accumulate Ca^{2+} during the insult (Hossman *et al*, 1985; Siesjö and Bengtsson, 1989).

It has been shown that restoration of normal levels of brain tissue Ca^{2+} occurs within 2-3 hours of recovery after global ischaemia (Simon *et al*, 1984; Deshpande *et al*, 1987). However, histological evidence shows that after a delay Ca^{2+} begins to rise in selectively vulnerable regions such as the cortex, hippocampus and striatum (Dienel, 1984). Stimulation of the Schaeffer collateral input to CA1 pyramidal cells elicits burst firing which is associated with transient decreases in extracellular Ca^{2+} and 8 hours after an episode of transient forebrain ischaemia the extent to which extracellular Ca^{2+} declines is considerably accentuated (Andine *et al*, 1988a). Recently Deshpande and colleagues (1987) have provided conclusive proof that tissue Ca^{2+} accumulation occurs at 48 - 72 hours after an ischaemic insult before neuronal necrosis of vulnerable cells, such as the CA1 pyramidal neurons, has occurred.

The axon-sparing dendrosomatic nature of global hypoxic-ischaemic cell death appears to correlate with localisation of glutamate receptors on apical dendrites and dendritic spines and of Ca^{2+} channels on central dendritic regions and soma (Johansen *et al*, 1984). Reduction of Ca^{2+} influx and accumulation, by lesioning of the glutamatergic input to CA1 pyramidal neurons, or by local application of the competitive NMDA antagonist AP7, corresponds to reduced ischaemic injury of CA1 neurons (Benveniste *et al*, 1988). The use of the dihydropyridine L-type Ca^{2+} channel antagonist nimodipine has been shown to reduce focal ischaemic injury, possibly through a reduction in Ca^{2+} influx although some researchers argue that the protective influence of this compound is through increased CBF (Uematsu *et al*, 1989; Greenberg *et al*, 1990; Teasdale *et al*, 1990). Flunarizine, another dihydropyridine antagonist, was found to reduce neuronal death in a canine model of global asphyxia (Espinoza and Parer, 1991). However, the entry of calcium via AOCC seems to be more significant since blockade

by NMDA receptor antagonists produces greater protection than direct calcium channel blockade (Siesjö, 1990). During and after global ischaemia Ca^{2+}-mediated phospholipase A_2 activation can attack cellular and organelle membrane phospholipids leading to free fatty acid release and therefore stimulation of prostaglandin synthesis which results in free radical formation that can also cause an attack on the structural integrity of the cell (Siesjö and Bengtsson, 1989; Vannucci, 1990; Siesjö, 1990 and see section 1.5.7). Calpain I activation mediated by Ca^{2+} results in proteolysis which, in combination with reduced protein synthesis, can also compromise the structural components of the cell (Raley-Susman and Lipton, 1989; Choi, 1990).

Not surprisingly Ca^{2+} accumulation has been shown to occur in the 7 day old rat brain during hypoxic-ischaemia and a secondary Ca^{2+} rise in selectively vulnerable areas was identified 72 hours after the insult (Stein and Vannucci, 1988). The fact that a hypoxic-ischaemic insult was found to enhance quisqualate-stimulated phosphoinositide hydrolysis thereby accentuating the rise in IP_3 particularly in the hippocampus and striatum is also of interest to Ca^{2+} homeostasis in the neonate (Chen *et al*, 1988). Flunarizine, a calcium channel antagonist, improved acute and chronic behavioural recovery and reduced cell death in hypoxic-ischaemia in the 21 day old rat (Gunn *et al*, 1989). Lidoflazine improved CBF when administered during resuscitation after global asphyxia in newborn lambs and in foetal sheep the administration of flunarizine prior to total cerebral ischaemia reduced cell death and improved recovery of electrocortical activity (Espinoza and Parer, 1991).

1.5.7 The Role of Free Fatty Acids and Free Radical Formation in Reperfusion Injury

Free radicals are highly reactive atoms or molecules which contain an uneven number of electrons in their outermost orbit (Vannucci, 1990). The generation of free radical elements occurs in a number of common metabolic reactions. For example, oxygen-free radicals ($^{\cdot}O_2^-$) are formed during electron transport within mitochondria or during prostaglandin synthesis. When formed $^{\cdot}O_2^-$ can combine with H^+ (derived from NADH or FADH) through the action of superoxide dismutase to form hydrogen peroxide (H_2O_2), and in the presence of Fe^{3+} or Cu^{2+}

the highly toxic superoxide anion ($^{\cdot}OH^{-}$) can be formed by combining $^{\cdot}O_2^{-}$ with H_2O_2. Oxygen-free radicals can initiate a chain of potentially destructive reactions, through lipid peroxidation, which attack the integrity of cellular membrane structures. Radical damage also occurs through injury of DNA, inactivation of enzymes and attack on structural proteins (Vannucci, 1990; Saugstad, 1990). Removal of free radicals from the cellular environment is achieved through a number of mechanisms including endogenous scavengers such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), cholesterol and thiol compounds, e.g. glutathione. The endoperoxidase and catalase enzymes protect the cell from the oxidising effects of H_2O_2.

The role of free radicals in hypoxic-ischaemic brain injury has recently gained prominence in particular with respect to their potentially destructive action during the reperfusion-reoxygenation phase. Raised levels of intracellular calcium during hypoxic-ischaemia stimulate the action of phospholipase A and phospholipase C (Siesjö, and Bengtsson, 1989; Siesjö, 1990; Vannucci 1990; Espinoza and Parer, 1991). The degradative action of these phospholipases on cellular and organelle membrane phospholipid components leads to a rise in the concentration of free fatty acids, particularly arachidonic acid, which persists long into the reperfusion period (Rehncrona *et al*, 1982; Gardiner *et al*, 1981; Bhakoo *et al*, 1984). The consequent disruption of membrane permeability exacerbates alterations of ionic fluxes. When oxygen delivery is re-established, the raised levels of arachidonic acid stimulate the cyclooxygenase and lipoxygenase pathways leading to metabolism of the arachidonic acid to eicosanoids including leukotrienes, prostaglandins and thromboxane A_2 (Choi, 1990; Espinoza and Parer ,1991; Siesjö, 1990). The cyclooxygenase and lipoxygenase systems release oxygen free radicals which can lead to the formation of the superoxide anion radical.

The site specific action of free radicals elicits a chain of destructive lipid membrane and DNA peroxidation thereby causing cell damage. However, the inhibitory action of oxygen free radicals on prostacyclin synthetase greatly influences circulatory events during the reperfusion phase. In the normal situation there exists a balance between prostacyclin, which is vasodilatory and inhibits platelet aggregation, and thromboxane A_2 which causes vasoconstriction and promotes platelet aggregation. This balance serves to maintain autoregulation and therefore vascular tone of the cerebral microcirculation (Siesjö, 1981).

Following hypoxic-ischaemia, inhibition of prostacyclin synthetase and production of thromboxane A_2 upsets the balance leading to vasoconstriction and blood cell aggregation. These are mechanisms that could ultimately cause a secondary hypoperfusion and therefore a secondary hypoxic insult (see Choi, 1990; Siesjö, 1990; Espinoza and Parer, 1991). In support of this hypothesis, the administration of the cyclooxygenase inhibitor indomethacin has been shown to improve postischaemic reperfusion in adult animals and humans (Espinoza and Parer, 1991). Secondary hypoperfusion has been demonstrated in many models of complete and incomplete ischaemia, and it has also been shown that secondary hypoperfusion impairs the initial recovery after the insult (Hossman and Kleihues,1973, Siesjö, 1981).

The energy deficit created during hypoxic-ischaemia results in ATP depletion and accumulation of AMP which can be further broken down to adenosine, inosine and hypoxanthine (Siesjö, 1981). Increases in adult and neonatal human CSF levels of hypoxanthine have been found 24 hours after hypoxia (Harkness and Lund, 1983), and hypoxanthine has also been shown to progressively accumulate, with increasing degrees of hypoxia, in foetal lambs (Thiringer *et al*, 1980; Thiringer *et al*, 1982). A calcium activated protease converts xanthine dehydrogenase into xanthine oxidase and when reflow is established xanthine oxidase utilises oxygen to convert xanthine to uric acid and in so doing produces oxygen-free radicals. Xanthine oxidase can be found in the endothelium of brain capillaries (Betz, 1985) and free radical damage may be one cause of blood brain barrier disruption and vasogenic oedema (Chan, 1984). Free radical production therefore influences many features during the reperfusion phase whether directly by destructive peroxidation mechanisms or indirectly by vasoconstriction, oedema formation and platelet aggregation.

Indirect support for free radical damage following hypoxic-ischaemia in adults was gained when the lipid peroxidation inhibitor 21-aminosteroid (U74006F) was shown to enhance survival 48 hours after transient forebrain ischaemia in gerbils and also reduce CA1 pyramidal cell death (Hall *et al*, 1988). The role of free radicals in perinatal hypoxic-ischaemia is a matter of some dispute since accumulation of free radicals in the neonatal piglet did not correspond with hypoxic-ischaemic neuronal damage (Armstead *et al*, 1988). The existence of foetal

tolerance to free radical damage could be related to transiently enhanced levels of antioxidants during the perinatal period and may also be one of the causes of foetal resistance to hypoxic-ischaemic brain injury (Saugstad, 1990).

This is, however, in conflict with data obtained from experiments using the model of hypoxic-ischaemia in the 7-day old rat (Rice *et al*, 1981). The xanthine oxidase inhibitors allo- and oxypurinol when administered prior to hypoxic-ischaemia reduced the extent of the post-insult oedema and also the extent of the infarct which suggests some influence of free radicals in the neonate (Palmer *et al*, 1990; Palmer *et al*, 1991). In addition, the lipid peroxidation inhibitor U74006F was shown to reduce oedema and neuropathological damage in the 7 day old rat (Bagenholm *et al*, 1991). It has been argued that the neuroprotective action of the glucocorticoid methylprednisolone in the 7 day old rat was also through inhibition of lipid peroxidation (Kalaych, 1991).

The use of inhibitors of prostaglandin synthesis and of radical-induced lipid peroxidation or of free radical scavengers may be of considerable benefit in the treatment of adult and neonatal hypoxic-ischaemic brain injury but the foetus may well have its own protective mechanisms (Siesjö, 1984; Kjellmer, 1991; Saugstad, 1990).

1.5.8 A Final Common Pathway Mediates Hypoxic-Ischaemic Cell Death: The Calcium Hypothesis

The previous sections have shown that several factors are likely to be involved in the process of hypoxic-ischaemic cell death. A recurrent theme is that most of these factors are linked, and set in motion a cascade of events causing initial, primary damage during the hypoxia-ischaemia and further secondary damage during the reoxygenation-recirculation phase. The initial oxygen deprivation results in insufficient ATP production leading to failure of ATP-dependent membrane functions which govern ion homeostasis. A slow efflux of K^+ causes presynaptic depolarisation and neurotransmitter release which triggers postsynaptic depolarisation thereby resulting in a rapid efflux of K^+ and influx of Na^+ , Cl^- and water.

Agonist operated and voltage sensitve calcium channels are activated which results in Ca^{2+} influx. ATP is not available to restore ion gradients or for reuptake of transmitters and ionic homeostasis is therefore lost (Siesjö and Bengtsson, 1989).

The common denominator is a loss of intracellular calcium homeostasis where excessive Ca^{2+} influx is added to by a loss of calcium extrusion and sequestration mechanisms. Calcium triggered activation of lipases, proteases and endonucleases leads to events including disruption of cellular and organelle membranes and fragmentation of DNA. Phospholipid degradation by calcium activated phospholipases leads to a rise in free fatty acids, particularly arachidonic acid. Since ATP is not available for the energy-requiring reassembly of molecules and molecular complexes membrane permability increases further exacerbating the loss of ionic homeostasis.

During reperfusion an initial reactive hyperemia restores oxygen delivery but the availability of oxygen, in combination with excess arachidonic acid and hypoxanthine, can now trigger events such as cyclooxygenase and lipoxygenase and xanthine oxidase driven reactions which result in the excessive production of oxygen free radicals. These attack the structural integrity of the cells and influence microcirculation. A secondary hypoperfusion after the insult reduces oxygen delivery just as cerebral oxygen consumption is beginning to recover and therefore an additional hypoxic-ischaemic insult is added to the primary trauma.

The situation in the hypoxic foetus is a little different because oxygen delivery to the brain is maintained by circulatory centralisation at the expense of peripheral organs (Jensen and Berger, 1991). If the insult is prolonged the centralisation mechanisms tend to fail and CBF falls thereby reducing oxygen supply. For brain injury to occur in the foetal situation, hypoxia must be accompanied by some element of cardiovascular failure which adds an ischaemic factor to the insult (Kjellmer, 1991; Espinoza and Parer, 1991). The mechanisms by which brain damage occur in the foetus may well be similar to those in the adult and action to prevent asphyxial injury must take account of the different factors. The key point which remains to be elucidated is at which stage is therapy appropriate. If damage occurs during the secondary reoxygenation-reperfusion phase this will leave an avenue for therapeutic intervention after the insult. However, if calcium entry during asphyxia sets in motion events which cannot be stopped then pretreatment strategies must be employed and this will involve significant

improvement in the identification of risk factors during pregnancy. In any case it seems likely that a combination of therapies will be appropriate and in one study in the foetal sheep, where free radical scavengers were combined with a calcium channel blocker and magnesium, post-asphyxial hypoperfusion was prevented and recovery of somatosensory evoked potentials was promoted (Thiringer *et al*, 1987; Kjellmer, 1991).

1.6 Hypoxia-Ischaemia Induced Changes in Learning and Memory

1.6.1 Adult Models of Ischaemic Memory Defects

Global and focal hypoxic-ischaemic insults in the adult human are known to cause amnesic syndromes that are distinct from those seen in alcohol-induced Korsakoff's syndrome or following bilateral temporal lobe resection (Zola-Morgan *et al*, 1986; Volpe and Hirst, 1983; Volpe *et al*, 1986). The amnesia induced by hypoxic-ischaemic episodes involves a loss of short term memory which can be expressed in the inability to freely recall learned material. Retrograde amnesia is rare, verbal and visual recognition is quite well preserved, and there is usually no other cognitive impairment (Zola-Morgan *et al*, 1986; Volpe and Hirst, 1983). The neuropathological correlate of this hypoxic-ischaemic induced amnesia is an extensive bilateral lesion of the CA1 field of the hippocampus (Zola-Morgan *et al*, 1986) but if damage extends to the subiculum and amygdala the amnesia may also be associated with dementia (Volpe and Petito, 1985).

The hippocampus is a brain region known to play a critical role in short-term memory formation (O'Keefe and Nadel, 1978; Squire, 1987; Squire, 1992). A bilateral hippocampal lesion in rats significantly impaired the performance of those rats in a place navigation task where the rats were trained to use extra-maze clues to locate a hidden platform in a water maze (Morris *et al*, 1982; Morris *et al*, 1990).

Transient forebrain ischaemia in adult rats is known to cause damage to the CA1 region of the hippocampus (Pulsinelli *et al*, 1982a; Smith *et al*, 1984b). Transient forebrain ischaemia has also been associated with deficits in spatial and working memory tasks (Auer *et al*, 1989; Volpe *et al*, 1984). In the place navigation task ischaemic rats took longer to learn the location of the platform, did not retain knowledge of the platform's position and were also particularly poor in a learning-set task involving 6 different platform positions (Auer *et al*, 1989; Voll *et al*, 1989). In the 8-arm radial maze task, where rats were expected to remember which particular arms were rewarded with food, the post-ischaemic rats also performed significantly worse than control animals (Volpe *et al*, 1984). The reference memory element of the tasks was only transiently effected but the trial-dependent working memory component showed a lasting impairment produced by the ischaemia (Volpe *et al*, 1984; Davis, 1987). In the more complex

working memory element, post-ischaemic rats frequently re-visited arms from which the food reward had already been taken. Similarly the greatest change in performance in the place navigation task was where the rats had to learn more than one platform position during a single testing session (Auer *et al*, 1989). The extrahippocampal damage, such as that in the dorsolateral striatum, is not likely to cause these memory impairments and interestingly performance in all of these tasks is improved if the rats received treatment known to reduce ischaemic hippocampal neuronal necrosis (Grotta *et al*, 1988; Voll *et al*, 1989; Block *et al*, 1990).

1.6.2. Perinatal Hypoxic-Ischaemia and Behavioural Defects

Hypoxic-ischaemic damage to the foetal or neonatal human infant causes predominantly motor impairments such as cerebral palsy and also seizures. However, mental retardation and, when the neuropathology is very limited, minimal brain dysfunction can also occur (Hill and Volpe, 1981; Towbin, 1971). The extent of the neuropathological damage and the neurological defect depends greatly upon the type and duration of the insult as well as on the age of the foetus or neonate.

Few studies have investigated the long-term behavioural consequences of perinatal hypoxia but the majority that have are associated with the induction of postnatal anoxia or hypoxia. Following the exposure to asphyxia at birth guinea-pigs have been shown to develop deficits in performance in complex-maze tasks (Windle and Becker, 1943; Bailey and Windle, 1959). The guinea-pigs were able to learn the rewarded sequence but always made more errors than the control animals.

Following exposure to repeated hypoxia during the last 14 days of gestation neonatal rats showed few early neurological defects but when they were adults they made significantly more errors on the difficult components of a Heb-Williams maze than control rats (McCulloch and Blackman, 1976). Rats exposed to intra-uterine asphyxia at birth did show early evidence of behavioural impairment (Bjelke *et al*, 1991). When tested at three weeks of age the rearing activity was lower than that of control animals and although normal locomotory behaviour was unaffected injection of apomorphine significantly enhanced the increased locomotory response

in the asphyxiated rats. Long-term behaviour was not tested in this model but it was argued that the early effects were related to increased numbers of dopaminergic neurons in the substantia nigra in conjunction with reduced numbers of CA1 and CA3 neurons in the hippocampus (Bjelke *et al*, 1991).

Post-natal anoxia induced on pnd1 by placing the rats in a 100% N_2 atmosphere for a minimum of 25 minutes caused a transient increase in sniffing and rearing activity between pnd20-pnd40 (Speiser *et al*, 1983; Herskowitz *et al*, 1983; Dell'Anna *et al*, 1991). This transient phase of hyperactivity, which was associated with exploration rather than locomotion, occurred between pnd7 to pnd20 if the anoxia was repeatedly induced on pnd2 and pnd4 (Nyakas *et al*, 1990).

Spatial memory tested in a maze in the early postnatal period, following exposure to anoxia on pnd1, was significantly impaired because the anoxic rats took longer to find a food cup than controls and also revisited sectors that had already been investigated (Dell'Anna *et al*, 1991). When spatial learning was tested in adult rats which had earlier been exposed to postnatal anoxia, the acquisition of the tasks was impaired and the working memory components were particularly impaired (Nyakas *et al*, 1990; Dell'Anna *et al*, 1991).

Post-natal anoxia has also been found to cause impairment in conditioned avoidance learning (Speiser *et al*, 1988; Nyakas *et al*, 1990) and in multiple choice discrimination tasks (Herskowitz *et al*, 1983). Both pre- and post-natal hypoxia and anoxia has been shown to affect complex behavioural tasks where the rat was expected to remember multiple cues and past-trial events at any one time which suggests that working memory tasks are particularly useful for investigating memory deficits in these models.

Pre- and post-natal anoxia causes a reduction in the number of cells in the CA1 region (Bjelke *et al*, 1991; Dell'Anna *et al*, 1991) and post-natal anoxia also causes a transient increase in hippocampal muscarinic acetylcholine receptors and a delayed lasting increase in ß-adrenergic receptors in the hippocampus (Herskowitz *et al*, 1983). Nigral dopaminergic cells are also increased following perinatal asphyxia but the duration of this increase has not been tested (Bjelke *et al*, 1991). The manipulation of a variety of neurotransmitter systems shown in these studies may have long lasting influences on behavioural development and particularly on

early learning and, later, more complex discrimination learning. Such a range of effects could have implications for attention disorders and hyperactivity associated with minimal brain disorder (Towbin, 1971; Wender, 1974; Wender, 1981) which suggests that these models could be suitable for the study of this disorder.

1.7 Aims and Objectives

The aim of this study was to develop a model of *in utero* hypoxia in the rat based on the original design reported by Magal and co-workers (1988). It was proposed that the investigation of several periods of uteroplacental vessel occlusion, conducted under anaesthesia, would provide a comprehensive survey of the metabolic alterations in the near-term foetal rat brain associated with this particular model of prenatal hypoxia. A pathological study of the hypoxic rat brains at several time points after the insult was undertaken to trace the development and pattern of any neuronal damage. In this way the value of this technique as a model of human perinatal hypoxic brain injury could be elucidated.

The final evaluation of the model involved a comprehensive survey of the growth and development of rats exposed to the longest period of hypoxia and when the rats reached 3 months of age a spatial memory task was used as a basic test of cognitive ability. Following the three main areas of investigation it should be possible to comment on the use and relative importance of this particular model with respect to the existing models of perinatal hypoxic-ischaemia. The direct comparison of brain tissue metabolite changes during and after the insult with a histological and developmental profile may also add to the debate on the mechanisms by which hypoxic-ischaemia produces long-lasting cerebral alterations in the perinatal animal.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 ANIMALS AND MATING PROCEDURE

The rat was the animal of choice for all experiments. All rats were bred in-house and kept on a 14-hour light, 10-hour dark cycle with free access to lab chow and water. Female Lister-Hooded rats at a minimum of 12 weeks of age were given a vaginal, saline smear at least 5 days a week. Rats in pro-oestrous were housed with a Lister-Hooded male overnight and smeared the following morning to detect the presence of sperm. If sperm were present this was taken to be day 1 of gestation. All pregnant rats were housed apart from non-mated cycling females but were not housed individually.

2.2 DEVELOPMENT OF SURGICAL PROCEDURE

2.2.1 Preliminary Method

The original surgical design (Magal *et al*, 1988) created a hypoxic insult in the *in utero* foetal rat without subjecting the dam to a hypoxic environment. The model as described originally has been modified quite considerably for optimal performance in this particular project.

The first attempt to reproduce the results of Magal *et al* (1988) used pregnant Lister-Hooded dams with pups at a gestational age of 21 (E21) days. Following induction of anaesthesia with 5% halothane in a 70%/30% nitrous oxide (N_2O) : oxygen (O_2) mixture, the dam was placed on a thermal barrier to restrict heat loss. The abdomen was cleaned with absolute alcohol and a midline abdominal incision was performed. Both uterine horns were exteriorised and one horn was selected for occlusion of the uteroplacental vessels.

The mesentery surrounding each uteroplacental vessel was cleared, using fine point forceps, and lengths of ED3 surgical thread were then passed around each vessel. The two ends of each piece of suture thread were gently teased into a 2cm length of small diameter plastic tubing. The thread was pulled taut so as to pull a small portion of the uteroplacental vessel up into the tubing, which led to the constriction of the

vessel, and the ends of the thread were then tied off. The time of the occlusion began when the first vessel was sealed. During the occlusion period the pups were covered with gauze and warm saline was dripped onto the gauze to keep the uterine wall warm and moist.

The first attempt at this particular method involved the removal of the tubing and the thread at the end of the occlusion period. The dam was then sutured and allowed to recover for 4 hours, to allow reperfusion of the occluded vessels, before being killed and the pups were delivered by Caesarean section. All attempts to resuscitate the hypoxic pups failed although the pups from the control, non-occluded horn were delivered and resuscitated with some ease. Further attempts using this method of occlusion looked at varying times of ligation from 10 to 30 minutes and also a comparison of the effects of delivering the pups either immediately after the occlusion or after a reperfusion period when the ligatures had been removed. Whichever variation was attempted the mortality rate was at best 75% but more usually 100%.

There were several limitations to the preliminary method which may be responsible for the high mortality rate; primarily, the method of ligation produced irreversible closure of the vessels. In addition, manipulation of the thread underneath each vessel often caused a rupture of the vessel.

2.2.2 Method of Occlusion

The next method of occlusion employed the use of standard metal artery clamps. Following exteriorisation of both uterine horns, mesentery was again cleared from each side of the uterine arteries. The extent of the occlusion was increased by occluding the main uterine artery for that particular horn with clamps placed just below the ovary and just above the branch point towards the cervix as well as around each uteroplacental vessel. It was deemed unnecessary to separate the artery and vein of each vessel since this would be time consuming and increased the risk of rupturing the vessels. During the occlusion period the control horn was kept inside the dam but the occluded horn was exteriorised and dampened with warm saline.

Only two attempts were made to clamp the main uterine artery because when the clamps were removed the arteries did not reopen. Further attempts involved the clamping of uteroplacental vessels only. Several clamp times were tested from a minimum of 10 minutes to a maximum of 57 minutes. If the clamp time was any longer than 30 minutes when the clamps were removed it was very difficult to see any blood reflowing in the vessels and clots could frequently be seen to be blocking the vessels. When the chest cavity of a foetal rat was opened after an occlusion of greater than 30 minutes the heart was motionless.

Having established that the maximum occlusion period was 30 minutes, 3 occlusion times were tested; 10, 20 or 30 minutes and during the occlusion period the anaesthesia was maintained. At this stage of the method development two possible procedures following the occlusion were decided upon. The first was the immediate delivery of the pups by Caesarian section even though the unclamped horn was anaesthetised. Some of the hypoxic rats appeared to be gasping upon delivery but between 80 and 100% died.

The alternative procedure was to remove the clamps and massage each vessel in an attempt to establish reflow. The horn was placed back inside the dam and the wound was sutured. The dam was taken off the anaesthetic and allowed to recover in a temperature regulated incubator (Model 002249, International Market Supply, UK) set at 37^{o}C. Following a period of recovery the dam was killed and the pups delivered as quickly as possible by Caesarian section. No pups survived with a recovery period of less than 10 minutes, and a recovery period of 15 minutes was also inadequate. Following a recovery period of 20 minutes the majority of control rats survived and many of the hypoxic rats were gasping although at best only 30-40% survived. No further improvement was achieved using recovery periods of greater than 20 minutes.

The limitation of this particular method was again shown in the lack of survival of the hypoxic pups which appeared to be related to the size of the clamps. A great many experiments had to be terminated because the physical manipulation of the vessels required to put these large clamps in place led to blood loss and upon removal of

the clamps it was difficult to establish reflow. The size of the clamps was therefore taken to be the major cause of failure in these experiments, and new, smaller clamps were used in all further experiments. The new clamps were angled Moria artery clips (Fine Science Instruments) which were made of light weight surgical steel and were a third of the size of the old clamps. All other aspects of the surgical procedure were maintained.

A slight improvement was noticed in that the smaller size of the clips caused less physical trauma and fewer ruptures occurred. When the clips were removed all of the vessels reopened and it was possible to see blood flowing. Despite the improvements mentioned the mortality rate for hypoxic pups was frequently 100% when using longer occlusion periods.

As flow transducers were not available it was not possible to determine the extent of the reduction of blood flow through the uteroplacental vessels. In addtion the size of the near-term foetal rat did not allow chronic physiological measurements and when attempts were made to collect arteriovenous blood from decapitated foetuses the samples were insufficient for accurate determination of blood gas measurements. At one stage attempts were made to place an oxygen sensitive electrode into the cortex of an exposed, insulated foetal rat brain whilst the rat was still attached to the umbilical cord and placenta. However, this was not successful and so accurate determination of the extent of the cerebral hypoxia could not be made.

2.2.3 Gestational age of the foetal rats

At this stage the procedure was changed by using pups at gestational age 22 days (E22) which is within 12-24 hours of normal vaginal delivery. The difference in size between pups at E21 and pups at E22 was quite surprising and the first experiments yielded a maximum mortality rate of 75% which was an improvement on the previous method using E21 rats.

Although we were satisfied that the most efficient method of clamping and the most appropriate age of pups was being used, it was still difficult to achieve rates of survival above 50% for the hypoxic pups. However, E22 rat pups were used in all future experiments.

2.2.4 Anaesthetic

The next change in protocol produced the most significant improvement yet. This was the change from using halothane to isoflurane for the induction and maintenance of anaesthesia. The respiratory depressant action of halothane had been a major drawback when attempting to resuscitate the pups. It was now possible to achieve resuscitation which resulted in regular independent gasping followed by abdominal breathing. The resuscitation took 10 minutes at most rather than a minimum of 15-20 minutes when using halothane. There were also many more pups gasping from delivery than were ever seen with halothane. Mortality rates also fell to a maximum level of 60% after the longest occlusion period.

2.2.5 Body temperature regulation

The maintenance of a constant body temperature is an essential component of any surgical technique. In experiments which aim to produce hypoxic-ischaemic brain damage temperature is particularly important due to the proposed neuroprotective effect of hypothermia (Busto *et al*, 1989).

The ambient temperature of the operating room for the preliminary experiments was $25 \pm 1^{o}C$. The dam was insulated using silver foil and the foil was shaped so as to enclose the exteriorised uterine horns. Saline, heated to $37 \pm 1^{o}C$, was dripped at regular intervals on to the exposed uterine horns but the pools of saline which collected around the uterine horns were not heated. A temperature probe inserted into the abdomen of a control rat after a 10 minute exposure of the uterine horns recorded a temperature of $31^{o}C$.

The ambient temperature was raised to 30 ± 2°C by using electric heaters. A thermal barrier (Vetko V-21) was used to regulate maternal temperature and the uterine horns were placed back inside the dam during the occlusion period. This method was tested by exposing foetal rats at certain times after the clips had been put on and placing a probe deep into the abdomen to measure body core temperature. The maternal temperature was maintained at 37 ± 1°C. Table 2.1 shows the change in temperature over a 40 minute period measured in one experiment. The temperature of both control and hypoxic rats was 35°C at the start of the experiment and fell to the unacceptably low level of 32-33°C. Heating through the use of an infra-red lamp was unsuccessful because this caused overheating of the dam and also it dried out the uterine walls. All further experiments used a room temperature of 32 ± 2°C in an attempt to maintain foetal body temperature.

TABLE 2.1 REGULATION OF FOETAL RAT BODY TEMPERATURE DURING INTRAUTERINE SURGERY

TIME (mins)	TEMPERATURE (°C)	
	CONTROL PUP	HYPOXIC PUP
0	35.0	34.5
10	33.5	33.4
20	32.5	33.5
30	33.2	33.5
40	33.8	-

2.3 A METHOD FOR THE INDUCTION OF AN IN UTERO HYPOXIC INSULT IN THE FOETAL RAT

The final protocol chosen as the most efficient procedure for this experiment uses very little of the original design. The only similarity to the original model is the use of Moria artery clips (Magal *et al*, 1988).

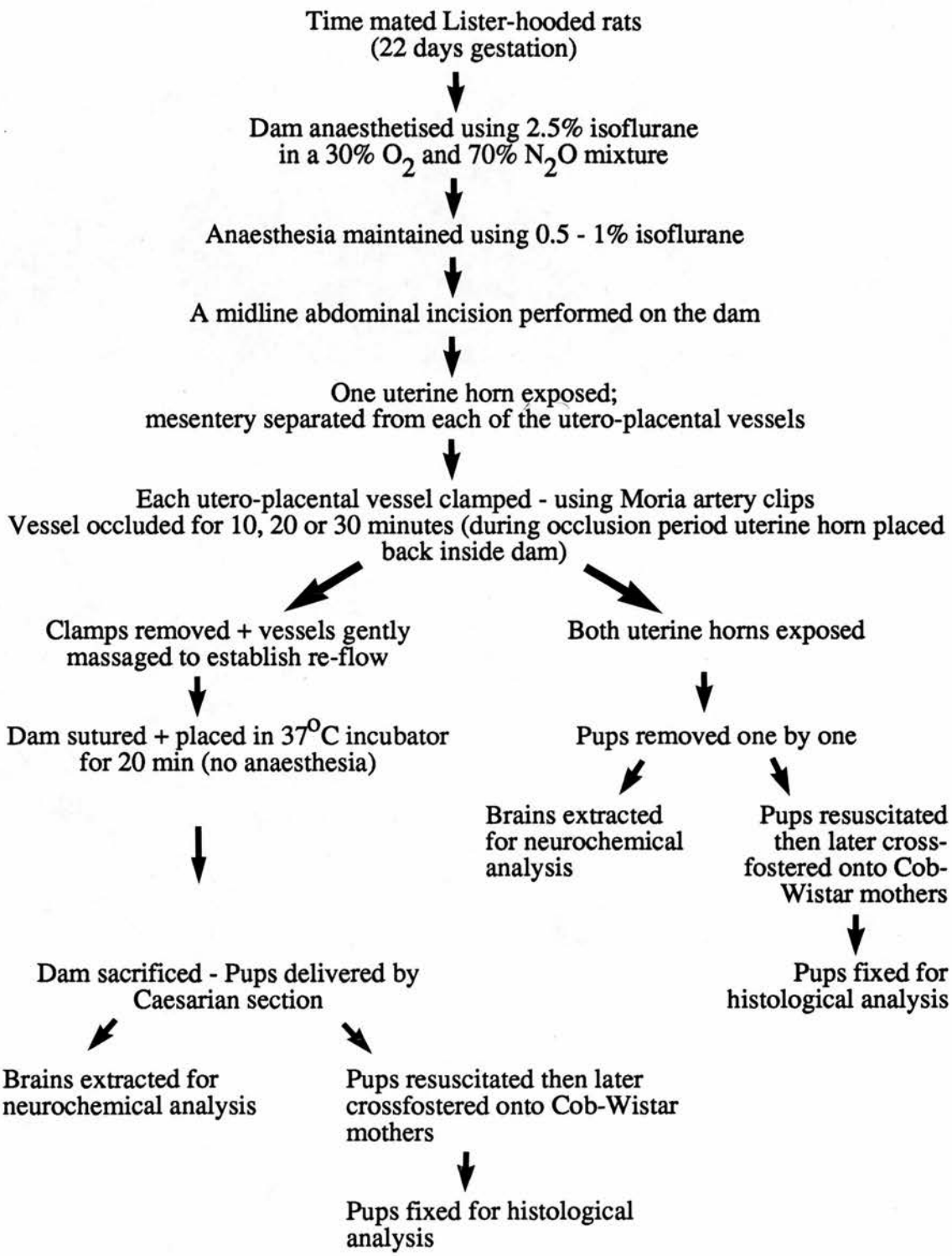
The flow diagram in figure 2.1 gives a pictorial representation of the fixed protocol. Pregnant Lister-Hooded rats with pups at gestational age 22 days were anaesthetised using 2.5% isoflurane in a mixture of 70% N_2O and 30% O_2. Following induction of anaesthesia the rat was transferred onto a thermal barrier and a nasal mask delivering 0.5 - 1% isoflurane was used for maintenance of anaesthesia. The ambient temperature was maintained at 32 ± 2°C and the maternal temperature was kept at 37 ± 1°C.

A mid-line incision was made along the abdomen and the uterine horns were exteriorised. If there were less than 4 pups in either horn the experiment was terminated and the dam sacrificed. If there appeared to be any gross abnormalities or deformities in any of the pups the experiment was stopped. The horn which appeared to have the most pups was selected for occlusion and the other control horn was placed back inside the dam.

The horn to be occluded was rested on the thermal barrier using a layer of gauze underneath the horn to prevent overstretching of the uteroplacental vessels. A small portion of mesentery was separated from either side of the vessels using fine tipped forceps. The separation was performed near the placenta since the branch points at the uterine artery side were more sensitive and vulnerable to mechanical damage. If at this stage any vessel was ruptured the experiment was terminated because experience showed that any blood loss resulted in the death of most of the pups before surgery could be completed.

Once a space had been cleared around each vessel a clip was placed on the vessel. The occlusion period was measured from the time the first vessel was clamped and all of the vessels were clamped within 30 ± 10 seconds. Once all of the vessels were clamped the horn was gently placed back inside the dam and care was taken not to dislodge any of the clips. The wound was then sutured and covered with gauze to reduce heat loss.

FIGURE 2:1 SURGICAL PROCEDURE



The above diagram is a pictorial representation of the surgical procedure used to produce an in utero hypoxic insult in foetal rats.

Three occlusion periods were used; ten, twenty or thirty minutes. After the set time the wound was reopened and either one of two procedures was followed. In the first the pups were delivered immediately by opening the horn and each amniotic sac. Time of delivery was taken at severence of the umbilical cord. The pups were delivered in a paired fashion such that the first hypoxic pup was removed from the lowest point on the uterine horn and the first control pup was removed from the corresponding point on the opposite horn. The following pups were removed sequentially moving up the uterine horns toward the ovarian end. Once delivered the pups could then be decapitated and their brains extracted for biochemical analysis (see Biochemical Analysis section 2.5) or else they were fully resuscitated and then cross-fostered onto lactating Cob Wistar dams for histological analysis at a later date (see histology; section 2.6).

Alternatively the clips were removed, the vessels gently massaged to establish reflow and then the uterine horn was placed back inside the dam. The wound was sutured using sub-cutaneous stitches. The dam was taken off the anaesthetic and placed in an incubator set at $37^{o}C$. 20 minutes was chosen as the time of recovery because this produced the greatest degree of survival of the hypoxic pups. Only one recovery time was used to restrict the number of experimental groups. After the recovery period the dam was killed and the pups were delivered by Caesarian section. The pups were used for biochemical or histological analysis as above.

There were 6 main experimental groups which involved occlusion of uteroplacental vessels:

i) 10 minutes occlusion followed by delivery of the pups

ii) 20 minutes occlusion followed by delivery of the pups

iii) 30 minutes occlusion followed by delivery of the pups

iv) 10 minutes occlusion + 20 minutes recovery + delivery of the pups

v) 20 minutes occlusion + 20 minutes recovery + delivery of the pups

vi) 30 minutes occlusion + 20 minutes recovery + delivery of the pups

There were also three control groups; the first, non-operated group, involved pups delivered by Caesarian section from an unanaesthetised dam which controlled for any possible mechanical interference from the surgery or the anaesthetic.

The second control (sham-operated-without recovery) group used rat pups from pregnant dams which were anaesthetised in the same way as for the above 6 groups. Following induction of anaesthesia a midline abdominal incision was made and both uterine horns exposed. The uterine horns were then placed back inside the dam and the wound sutured. Anaesthesia was maintained for 25 minutes and the pups then delivered by Caesarian section. The third control (sham-operated-with recovery) group followed the same procedure as the second except that after the 25 minutes the anaesthetic was turned off and the dam allowed to recover for 20 minutes and then the dam was killed and the pups were delivered. These two groups controlled for interference caused by the physical manipulation of the uterine horns and surgical trauma but not for the anaesthetic. 25 minutes of anaesthesia was chosen as the suitable midpoint and to represent the total time that the animals occluded for 20 minutes would be exposed to anaesthetic.

Table 2.2 shows the fluctuations in both maternal and foetal rat temperatures recorded in one experiment when using the surgical procedure described above. In this example foetal rat body temperature was maintained within an acceptable range of 34-38^{o}C.

TABLE 2.2 THE CHANGE IN MATERNAL AND FOETAL RAT BODY TEMPERATURE DURING IN UTERO SURGERY

TIME	TEMPERATURE (^{o}C)		
(mins)	MATERNAL	CONTROL RAT	HYPOXIC RAT
0	36.5	38.3	34.3
10	37.0	35.8	35.5
20	37.0	38.3	37.0
25	37.5	34.9	34.7
30	37.5	34.6	34.6

2.4 RESUSCITATION AND CROSS FOSTERING OF THE PUPS

2.4.1 Resuscitation

All of the pups, once delivered, had to be resuscitated manually. In the first instance this involved clamping and then cutting the umbilical cord. The pups were cleaned using gauze, a gentle chest massage promoted gasping and the pups were breathed on in order to keep them warm during the resuscitation period.

Only those pups which were gasping on delivery could be resuscitated and several methods were tried to promote maximal survival. Massage of the chest did not produce sufficient physical stimulation and so the pups were given a gentle shake to promote coughing up of fluid. When the pups were placed under a pure oxygen stream the skin turned pink but the oxygen did not promote gasping and the air stream tended to cool the pups.

The most reliable method of resuscitation involved constant handling and wiping away of the coughed up fluid. It seemed to be a general rule that if a pup was going to survive independent gasping had to be established within 5-10 minutes. Any pups which took longer than 10 minutes to begin independent gasping were discarded since it was presumed they would be exposed to an additional period of hypoxia.

Once independent gasping had been established the pups were placed in an incubator set at 37°C. A secondary period of respiratory depression often occurred at around 20 minutes after delivery. This may be a natural phenomenon but in any case the pups had to be constantly monitored even after regular abdominal breathing had begun.

2.4.2 Cross-fostering Procedure

All pups required for histological or behavioural analysis beyond the age of 4 hours were cross-fostered. Cob Wistar dams were used as foster mothers because experience showed that Lister-Hooded mothers tend to reject foster pups. The first attempts at cross-fostering used dams which had delivered their litters naturally two days before the operated pups were delivered. The Cob Wistar litter was cut back to between 2 and 4 pups.

The foster mother was removed from the cage at least one hour before the operated pups were to be put in. The cage was not cleaned after the litter was born to mask the odour of the foreign offspring and the Lister-Hooded pups were rubbed with damp debris from the dirty cage and then mixed among the Cob Wistar pups. Around 30 minutes after placing the pups in the cage the mother was returned to the cage and then left undisturbed in the dark for at least 2 hours.

The first attempts at cross fostering were very unsuccessful. By the following morning most of the fostered pups were found dead or were missing and therefore presumed to be eaten by the mother. If, however, the host mother's litter was only one day old she was more likely to accept the foreign offspring. Cob Wistar dams were therefore time-mated such that they would deliver one day before the surgery. The Cob Wistar pups were still a great deal larger than the Lister-Hooded pups even though they were just one day older so the Cob Wistar litter was reduced to 2 male pups and a maximum of 6 Lister-Hooded pups were added to the litter. To further mask the odour of the foreign pups the Cob Wistar pups were encouraged to urinate and the urine rubbed over the Lister-Hooded pups.

Identification of pups was a major problem - it was preferential to place all the pups to be cross fostered in the same cage because certain mothers were more attentive than others. If the litters were separated into different cages then the two sets of pups did not grow and gain weight concurrently. Thus, both control and hypoxic pups were placed with the same dam. An indelible marker was used to mark the tails of the hypoxic pups but the most attentive mothers tended to wash the majority of the ink off.

Removing the end of the tail proved to be a reliable method of identification but, because head-to-tail measurements were made for full assessment of growth this method was also unsuitable. Another method of identification was the removal of one of the toes soon after delivery and different toes could be removed to ensure identification of individual pups. The toe was clamped to halt the blood supply and then quickly removed using a small pair of spring action scissors. The method appeared to cause little distress to the pup and only a very small blood loss. However, a great many pups were dead or missing the next day. The surviving rats suffered no apparent disability as a result of this method but the levels of mortality were considered unacceptable.

The only method which did not affect mortality or measurements was the marking of pups using indelible ink. Different colours were used to distinguish individuals and pups were checked twice daily to replace any marks which had been removed by attentive mothers. The pups were marked by an attendant from the animal house such that all future observations could be performed blind.

2.5 BIOCHEMICAL ANALYSIS OF FOETAL BRAIN TISSUE

The preparation of foetal brain tissue was based on the method described by Lowry and Passoneau (1972). After completion of the occlusion period or recovery period the dam was sacrificed. The abdominal wound was re-opened, both uterine horns were exposed and one pup from each uterine horn was removed. The umbilical cord of each rat was cut, the rat was decapitated and the whole brain was removed using a small spatula.

The following provides a step-wise description of the procedure used for the preparation of brain tissue from each rat.

1. The brain was put into a 1ml Eppendorf tube which containing 300µl perchloric acid ($HClO_4$) at -10 ± 1°C.
2. The brain was homogenised and the tube was left on ice.
3. 10 minutes after homogenisation, 1ml of deionised water at 4°C was added to the tube. The tube was shaken and then left on ice for 5 minutes with occasional shaking.
4. The Eppendorf tube was centrifuged at 10000g for 2 minutes at 4°C.
5. 1ml of the supernatant was withdrawn and 400µl of 2M potassium hydrogen carbonate ($KHCO_3$) at 4°C was added to neutralise the acid. The tube was left on ice for 15 minutes.
6. The tube was then centrifuged for 2 minutes at 10000g.
7. 200µl aliquots of the supernatant were placed in 500µl Eppendorf tubes, the tubes were placed on dry ice and, when frozen, stored at -70°C.

The dissection of each brain took no longer than 15 seconds from removal from the uterine horn and the severance of the umbilical cord was taken as the start point. One pup from each uterine horn was killed at zero minutes, then two more were killed at one minute after severance of the cord, after 60 minutes and when possible after 180 minutes. This provided brain tissue samples for both control and hypoxic foetal rats at 0, 1, 60 and 180 minutes after the surgery was completed.

2.5.1 The Measurement of Brain Tissue Lactate

The level of brain tissue lactate in foetal rats was assayed using the Sigma diagnostic kit (826-U.V.). The assay is based on the reaction shown below which is catalysed by the enzyme lactate dehydrogenase.

$$\text{Lactic Acid} + NAD^+ \text{ ------> } \text{Pyruvic Acid} + NADH$$

In the presence of lactate the addition of excess NAD^+ results in the formation of NADH which has a higher absorbance at 340nm than NAD^+. The increase in absorbance, measured using a Cecil Ultra-violet spectrophotometer was the indication of the concentration of brain tissue lactate present in each sample.

Procedure for the measurement of brain tissue lactate

1. A standard curve was prepared using lactate solutions in the range 0.04mg/ml to 0.2mg/ml. The solutions were kept on ice until required.
2. The glass vials used for each individual assay were labelled and kept on ice until required.
3. Brain tissue samples were removed from the -70°C freezer and allowed to defrost at room temperature.
4. The NAD^+ reagent mixture was prepared as follows: 5mg NAD^+/ml of 0.6M glycine buffer (pH 9.2), 2.0 ml of deionised water/ml buffer and 50µl of lactate dehydrogenase/ml buffer. The mixture was shaken gently and kept on ice until required.
5. 1.4ml of NAD^+ reagent mixture was pipetted into each vial. The reaction was started by the addition of 100µl of a $HC10_4$ blank, lactate standard solution or brain tissue sample. Each glass vial was shaken well then placed in a water bath, set at 25°C, for 30 minutes.
6. An ultra-violet spectrophotometer (Cecil Series 2 : CE292) was set to 340nm and the set-zero position was checked and corrected if necessary.
7. Following the 30 minute incubation the absorbance of each sample was measured using the $HC10_4$ blank as the zero reference.

2.5.2 Fluorimetric Analysis of Energy Metabolites in Brain Tissue Samples

Certain energy metabolites were assayed using a Shimadzu Fluorimeter (RF-5000) because the high sensitivity of this instrument can detect concentrations of energy metabolites in the range 0.1 - 10μM. Fluorescence of the reduced pyridine nucleotides formed the basis of all the assays used (Lowry and Passoneau, 1972). The fluorescence of the pyridine nucleotides is sensitive to pH such that a pH below 7 was avoided because of the instability of the reduced nucleotides and a pH above 10.5 was avoided to prevent conversion of oxidised nucleotides into fluorescent products. There is a large negative temperature coefficient of around 1.6% for the fluorescence of pyridine nucleotides and so all standards and samples were measured in a temperature controlled chamber set at 25^{o}C.

The fluorescence of NADH/NADPH was detected using an emission wavelength of 420nM and an excitation wavelength of 340nM. All samples were measured in 1ml silica glass cuvettes (BDH Ltd., UK) which were cleaned using absolute alcohol and rinsed with distilled water. The background fluorescence of enzymes was kept to a minimum by using very small volumes (1-5μl) in a total assay volume of 1ml.

2.5.3 The Measurement of Brain Tissue Glucose

This assay was taken from the method described by Lowry and Passoneau (1972) and is based on the following reactions:-

a) Glucose + ATP -> Glucose-6-phosphate + ADP

b) Glucose-6-phosphate + $NADP^{+}$ -> 6-phosphogluconolactone + NADPH + H^{+}

Reaction a) is catalysed by the enzyme hexokinase and reaction b) is catalysed by glucose-6-phosphate dehydrogenase. In the presence of these enzymes NADPH was formed and the level of fluorescence in the assay mixture increased. The change in fluorescence was used to calculate the concentration of glucose in each sample.

Procedure for the measurement of brain tissue glucose

1. The reagent mixture consisted of 50mM Tris-HCl buffer (pH 8.1), 1mM $MgCl_2$, 0.5mM dithiothreitol, 300μM ATP and 30μM $NADP^+$. The mixture was shaken and left on ice until required.

2. The enzymes were prepared using Tris-HCl buffer to dilute stock solutions.

   a) Hexokinase

   The stock solution of 140 units/mg (2mg/ml) was diluted to 28U/ml by adding 90μl of Tris-HCl buffer to 10μl hexokinase. A final reaction concentration of 0.14U/ml was achieved by adding 5μl of diluted hexokinase to 1ml of reagent.

   b) Glucose-6-phosphate dehydrogenase

   The stock solution of 140U/mg (1mg/ml) was diluted to 7.0U/ml by adding 190μl of Tris-HCl to 10μl of glucose-6-phosphate dehydrogenase. As glucose-6-phosphate was not being assayed this enzyme was added to the reagent mixture. 3μl/ml of diluted enzyme was added to the reagent to achieve a final concentration of approximately 0.02U/ml. The enzymes were stored on ice until required.

3. Glucose standard solutions (50μM-375μM) were prepared and 50μl of standard was added to each assay to achieve a standard curve between 1.0μM and 7.5μM.

4. Brain tissue samples were removed from the -70°C freezer and defrosted at room temperature.

5. Glass vials for each standard and sample were labelled and kept on ice. 980μl of reagent mixture was added to each vial followed by 20μl of $HC10_4$ blank, glucose standard or tissue sample. Each vial was vortex mixed and stored on ice until required. 5 minutes before measurement the vial was taken off the ice and left at room temperature.

6. The sample to be assayed was placed in a 1ml cuvette and then the cuvette was put into the measuring chamber of the fluorimeter. The baseline was left to settle to allow for temperature equilibration of the sample to 25°C. The

fluorescence was recorded (blank) and then 5µl hexokinase was added to the sample, the cuvette was shaken gently and put back in the chamber. 10 minutes later the final fluorescence was measured (glucose measurement).

2.5.4 The ATP and Phosphocreatine Assay

This assay was based on the 3 reactions shown below:-

a) Phosphocreatine + ADP ----> Creatine + ATP

b) ATP + Glucose ----> ADP + Glucose-6-phosphate

c) Glucose-6-phosphate + $NADP^+$ ---> 6-Phosphogluconolactone + NADPH + H^+

Reaction a) is catalysed by the enzyme creatine kinase, reaction b) is catalysed by hexokinase and reaction c) is catalysed by glucose-6-phosphate dehydrogenase.

The level of ATP was measured using the increase in fluorescence 10 minutes after the addition of hexokinase and the level of phosphocreatine (Pcr) was measured 15 minutes after the addition of creatine kinase (Lowry and Passoneau, 1972).

Procedure for the measurement of ATP and PCr

1. The reagent mixture consisted of 50mM Tris-HCl (pH 8.1), 1mM $MgCl_2$, 74µM dithiothreitol and 100µM glucose. The mixture was shaken and stored on ice until required.
2. A 10mM ADP solution was prepared and stored on ice until required.
3. The enzyme solutions were prepared from stock solutions.
   a) Glucose-6-phosphate dehydrogenase

      The stock solution of 140 units/mg (1mg/ml) was diluted to 14U/ml by adding 90µl of 50mM Tris-HCl to 10µl of stock glucose-6-phosphate dehydrogenase. This assay was not measuring glucose-6-phosphate and so 2µl/ml of diluted enzyme was added to the reagent mixture to provide a final assay concentration of 0.03U/ml.

b) Hexokinase

The stock solution of 140U/mg (2mg/ml) was diluted to 140U/ml by adding 50µl 50mM Tris-HCl to 50µl of the stock hexokinase. A final assay concentration of 0.28U/ml was achieved by adding 2µl to each 1ml sample.

c) Creatine kinase

Creatine kinase was freshly made for each assay by dissolving 2.0mg of the 350U/mg stock powder in 233µl of 50mM Tris-HCl to provide a stock solution of 3000U/ml. This stock solution was diluted to a final assay concentration of 9U/ml by adding 3µl to each 1ml sample.

Each enzyme was stored on ice until required.

4. a) The solutions for the ATP standard curve were prepared from a stock solution of 10mM ATP. ATP solutions (25 - 250µM) were prepared to provide a final standard curve between 0.5 - 5µM. The solutions were kept on ice until required.

   b) The solutions for the PCr standard curve were also prepared from a freshly made stock solution of 10mM PCr. PCr solutions (50 - 250µM) were prepared to provide a standard curve between 1-5µM, and the solutions were stored on ice until required.

5. The brain tissue samples were retrieved from the -70^{o}C freezer and defrosted at room temperature.

6. The glass vials required for each standard and sample were labelled and placed on ice. 980µl of reagent mixture was added to each vial followed by 20µl of $HClO_4$ blank, ATP standard, PCr standard or brain tissue sample. Each vial was vortex mixed and then kept on ice until required.

7. Each glass vial was removed from the ice 5 minutes before the sample was poured into a silica cuvette and then placed in the fluorimeter chamber which was heated to 25^{o}C. The fluorescence was recorded (blank) and 2µl hexokinase was added to the cuvette. The cuvette was gently shaken and then placed back

into the measuring chamber. 10 minutes later the fluorescence was read (ATP measurement) and 3µl of creatine kinase and 10µl of 10mM ADP were then added to the cuvette, the cuvette shaken and put back into the measuring chamber of the fluorimeter. 15 minutes later the final fluorescence was recorded (PCr measurement).

2.5.5 The ADP and AMP assay

The assay used to measure the concentrations of brain tissue ADP and AMP was taken from the method described by Lowry and Passoneau (1972). The assay was based on the following 3 reactions:-

a) AMP + ATP ----> 2.ADP

b) ADP + Phosphoenolpyruvate ----> ATP + Pyruvate

c) Pyruvate + NADH + H^+ ----> Lactate + NAD^+

Reaction a) is catalysed by the enzyme myokinase, reaction b) is catalysed by pyruvate kinase and reaction c) is catalysed by lactate dehydrogenase.

The level of ADP in each sample was measured using the fall in fluorescence after the addition of pyruvate kinase and the level of AMP was measured using the further decrease in fluorescence after the addition of myokinase.

Procedure for the measurement of ADP and AMP

1. The reagent mixture was prepared using 50mM imidazole buffer (pH 7.0), 2mM $MgCl_2$, 75mM KCl, 20µM phosphoenolpyruvate, 5µM ATP and 10µM NADH. The reagent was shaken well and kept on ice until required.
2. The enzyme solutions were prepared from stock solutions.
   a) Lactate dehydrogenase

      The stock solution of 1000 units/ml was diluted to 200U/ml by adding 80µl of imidazole buffer to 20µl lactate dehydrogenase. Pyruvate was not assayed in the samples and so 3µl/ml of diluted lactate of dehydrogenase was added to the reagent to achieve a final assay concentration of 0.6U/ml.

b) Pyruvate kinase

The stock solution of 200U/mg (2mg/ml) was diluted to 200U/ml by adding 50µl of imidazole buffer to 50µl of pyruvate kinase. To achieve a final assay concentration of 1U/ml, 5µl of diluted enzyme was added to each 1ml assay.

c) Myokinase

The stock solution of 360U/mg (2mg/ml) was diluted to 180U/ml by adding 60µl of imidazole buffer to 20µl of myokinase. A final assay concentration of 0.36U/ml was achieved by adding 2µl of diluted enzyme to each 1ml assay.

All of the enzymes were stored on ice until required.

4. a) The ADP standard curve was prepared by making ADP solutions (50-250µM) from a 10mM ADP stock. The addition of 20µl standard to 980µl reagent achieved a standard curve between 1-5µM. The solutions were stored on ice until required.

b) The AMP standard solutions (12.5-125µM) were prepared from a 10mM stock solution. The standard curve was measured by adding 20µl of standard to 980µl reagent to provide 0.25 - 2.5µM AMP. The solutions were kept on ice until required.

5. The frozen brain tissue samples were defrosted at room temperature.

6. The glass vials were labelled and placed on ice. 980µl of reagent was pipetted into each vial followed by 20µl of $HClO_4$ blank, ADP or AMP standard or brain tissue sample. The vials were vortex mixed and stored on ice until required.

7. 5 minutes before measurement the vial was taken off the ice. The sample was poured into a silica cuvette and the cuvette was placed in the fluorimeter measuring chamber which was heated to 25°C. As soon as the baseline reading had settled the fluorescence was recorded (blank) and 5µl of pyruvate kinase was then pipetted into the cuvette, the cuvette shaken and placed back in the

measuring chamber. Not less than 7 minutes later the fluorescence was recorded (ADP measurement), and 2µl myokinase was then added to the assay. 10 minutes later the final fluorescence was recorded (AMP measurement).

2.5.6 Quantitation of Brain Tissue Concentrations of Metabolites

The procedure followed for the preparation of the brain tissue samples involved several dilution steps and these were taken into account for the calculation of accurate concentrations of metabolites present in each sample. To X mg of brain tissue was added 300µl of $HClO_4$ then 1000µl of water. A total volume of 1.3ml acid was neutralised using 0.52ml $KHCO_3$ to provide a final volume of 1.82ml. 1.82 was used as the overall dilution factor when calculating brain energy metabolite concentrations.

Data from the standard concentrations of each metabolite was analysed using a computer-operated Fitkit programme ((c) D. Barlow). With the exception of glucose all data fitted best to the straight line equation $y = mx + c$, where y = the absorbance/fluorescence and x = the concentration of each standard solution measured. Table 2.3 summarises the results obtained when $y = mx + c$ was applied to the standard measurements.

TABLE 2.3 SLOPE GRADIENTS AND AXIS INTERCEPTS OBTAINED FROM STANDARD CURVE ANALYSES

METABOLITE	m	c
Lactate	44.76	0
ATP	6.281	0
PCr	10.381	10.022
ADP	8.290	0
AMP	16.090	0

When the formula x = y-c/m was applied the level of metabolite in each sample (x) could be calculated. (y = level of absorbance/fluorescence).

The glucose standards best fitted a curve and thus the calculation of the slope coefficient and axis intercept was based on the equation y = mx/x+k. This produced a slope value of m = 96.637 and an intercept of k = 8.012. The level of glucose in each sample was calculated using x = -yk/y-m. The standard curves for each metabolite are shown in figure 2.2.

To calculate the exact concentration of metabolite present in each foetal rat brain all sample dilution factors and brain tissue weights were taken into account.

1) Lactate

$$\frac{x \,.\, 1.82 \,.\, 10 \,.\, 1000}{Wt \,.\, 90} = \text{[Brain tissue lactate] nmols/mg}$$

where x = concentration of lactate in sample (µg/0.1ml)

1.82 . 10 = the dilution factor (100µl sample in 1ml)

1000 = conversion factor (µmols to nmols)

Wt = weight of rat brain (mg)

90 = molecular weight of lactate

2) Glucose, ATP, PCr, ADP and AMP

$$\frac{x \,.\, 50 \,.\, 1.82}{Wt} = \text{[Brain tissue metabolite] nmols/mg}$$

where x = concentration of metabolite in sample (µM)

50 = dilution factor (20µl sample in 1000µl assay)

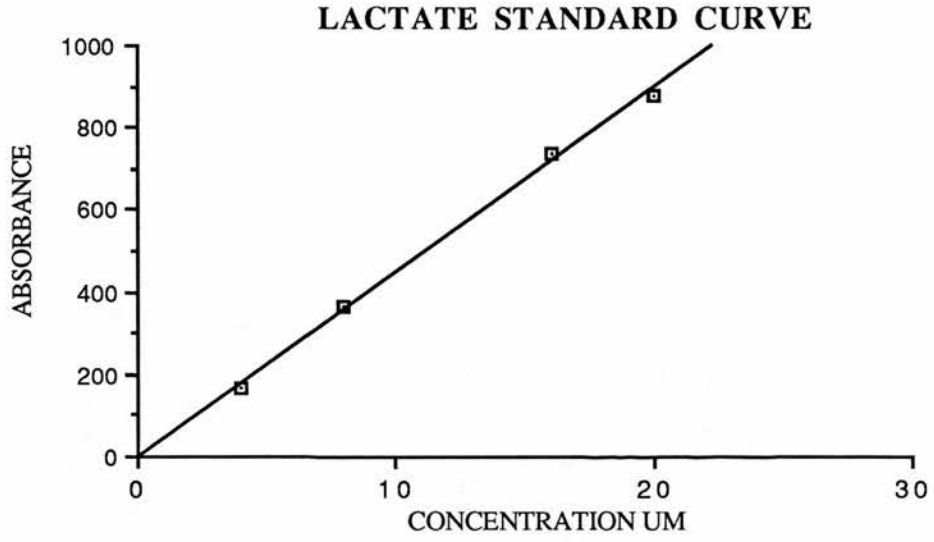
1.82 = dilution factor from tissue preparation

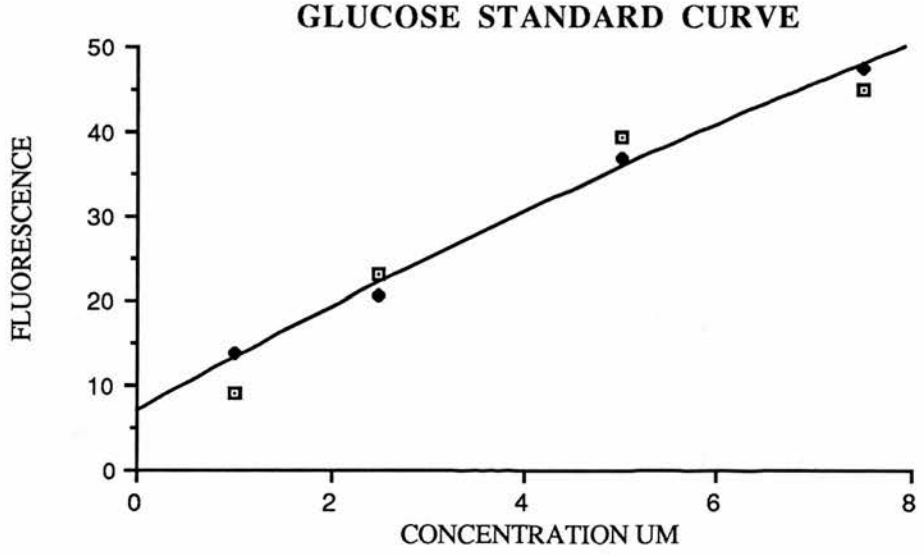
Wt = weight of the rat brain (mg)

FIGURE 2.2a STANDARD CURVES FOR THE BIOCHEMICAL ASSAYS

The three diagrams show the standard curves generated after the assay of known concentrations of metabolites. The equations derived from these standard curves were used to calculate the exact concentrations of metabolites in brain tissue samples (see text). The correlation coefficient (r) for lactate was 1.000, r for glucose was 1.000 and r for ATP was 0.999. The calculations were made using the Fitkit programme (c. D.Barlow).

GURE 2.2 a


LACTATE STANDARD CURVE
ABSORBANCE
1000
800
600
400
200
0
0
10
20
30
CONCENTRATION UM


GLUCOSE STANDARD CURVE
FLUORESCENCE
50
40
30
20
10
0
0
2
4
6
8
CONCENTRATION UM

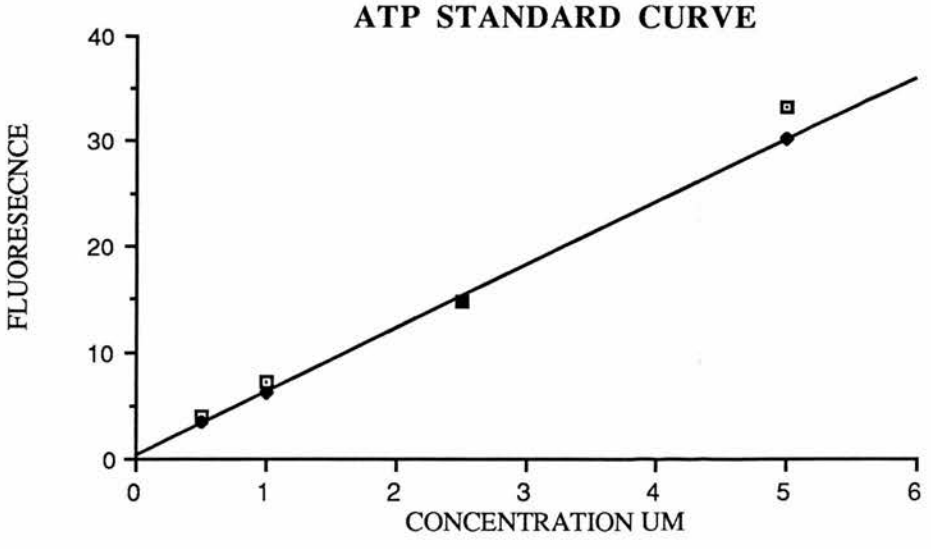
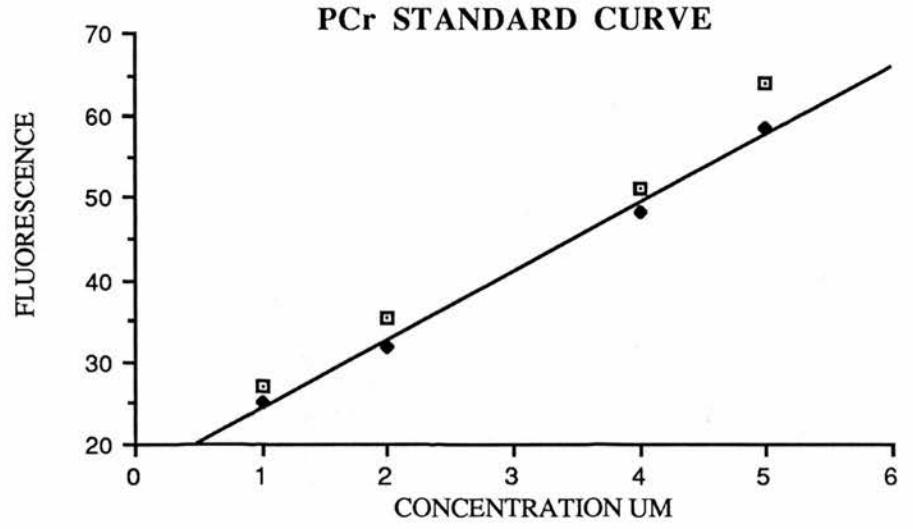
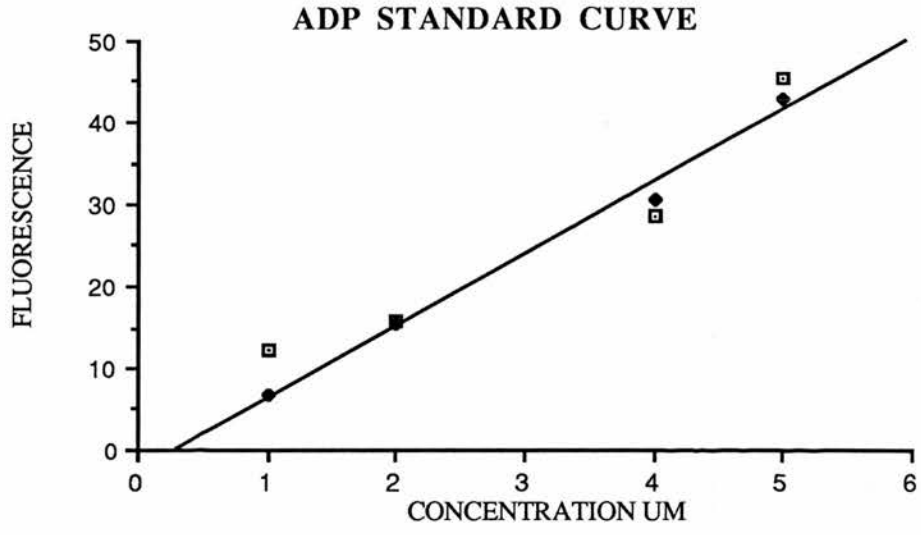

ATP STANDARD CURVE
FLUORESECNCE
40
30
20
10
0
0
1
2
3
4
5
6
CONCENTRATION UM

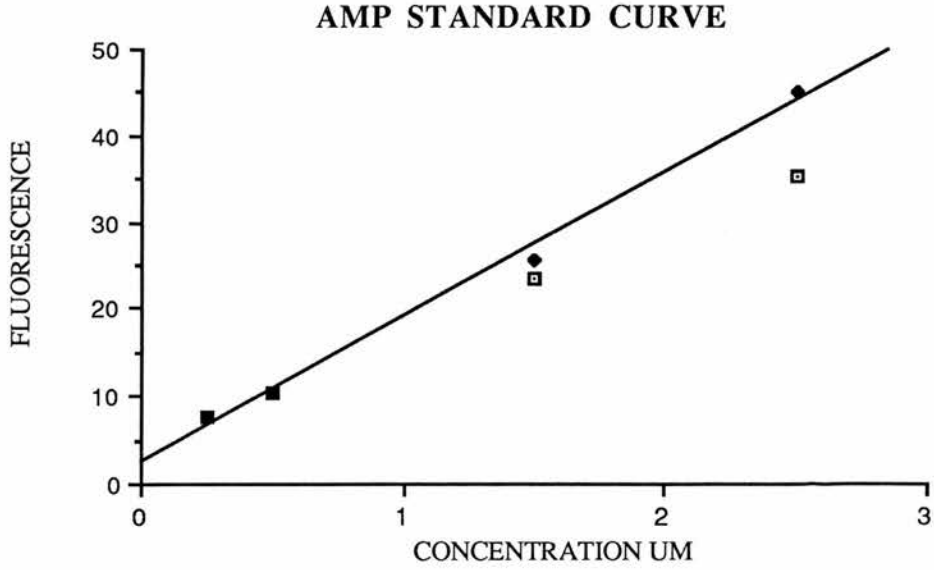
FIGURE 2.2b STANDARD CURVES FOR THE BIOCHEMICAL ASSAYS

The three diagrams show the standard curves generated after the assay of kno wn concentrations of metabolites. The equations derived from these standard curves were used to calculate the exact concentrations of metabolites in brain tissue samples (see text). The correlation coefficient (r) for PCr was 0.994, r for ADP was 0.993 and r for AMP was 0.988. The calculations were made using the Fitkit programme (c. D.Barlow).

'IGURE 2.2 b


PCr STANDARD CURVE
FLUORESCENCE
70
60
50
40
30
20
0
1
2
3
4
5
6
CONCENTRATION UM


ADP STANDARD CURVE
FLUORESCENCE
50
40
30
20
10
0
0
1
2
3
4
5
6
CONCENTRATION UM


AMP STANDARD CURVE
FLUORESCENCE
50
40
30
20
10
0
0
1
2
3
CONCENTRATION UM

2.6 HISTOLOGICAL ANALYSIS OF NEONATAL RAT BRAINS

2.6.1 Development of a Procedure for the Fixation and Processing of the Perinatal Rat Brain

There are few, tried and tested methods for a consistent fixation procedure for neonatal rat brains. As the water content of neonatal brains is very high, sectioning using freezing techniques was likely to cause artefacts.

The usual method of fixation in this laboratory involved transcardial perfusion with heparinised saline (40µl of 4000U/ml heparin in 100ml 0.9% saline) followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer at pH 7.4 with 1.25% gluteraldehyde. This fixative was used for preliminary experiments on foetal rats at 4 hours of age.

The rats were anaesthetised using diethyl-ether, the thorax opened and a needle inserted into the left ventricle. Heparinised saline (at 37^{o}C) was infused at 1ml/minute for 2 minutes and then 4% paraformaldehyde in sodium phosphate buffer with 1.25% glutaraldehyde was infused for 5 minutes. The perfused rat was decapitated, the brain removed, placed in fixative and then stored at 4^{o}C overnight.

The following day the brain was processed for wax-embedding. This involved dehydration of the brain in 50% absolute alcohol for 2 washes of 15 minutes each, followed by 75%, 80%, 90%, 95% and 100% absolute alcohol all for 2 washes of 15 minutes. At first there were fewer changes of alcohol but dehydration through 75% to 100% with 4 different grades of alcohol produced excessive shrinkage of the fixed brains so the more gradual dehydration was used to reduce shrinkage. The brains were placed in xylene for 15 minutes and then into fresh xylene until they became translucent, after around 40-45 minutes.

The brains were then taken through 5 changes of molten paraplast wax (Sherwood Medical Ltd, UK) at 60^{o}C. After the 5 changes of wax the brains were left overnight in an incubator at 60^{o}C. The brains were embedded in fresh wax using plastic moulds. After the wax formed a firm layer on the surface of the mould the entire mould was covered with cold water and left for several hours.

The wax was removed from the mould, trimmed until the brain was almost visible and the block was placed onto a heated brass chuck. The chuck was placed into a microtome and ribbons of sections at 6μm were cut from the block. Each ribbon of sections was floated out on heated water at 50-54^{o}C and then mounted onto albumen coated slides. The sections were left to dry overnight in a 37^{o}C incubator.

The next day it was not even necessary to stain the sections to see how poor the quality was. The brains were shrunken and mis-shapen with disproportionately large lateral ventricles and each section was badly cracked. Those sections which were stained, using the nissl stain cresyl fast violet, showed a higher proportion of dark-cell artefacts associated with inadequate fixation and post-fixation trauma (Cammemyer, 1961 and 1978).

2.6.2 Fixation

Alternative methods of fixation were attempted; immersion fixation produced severely shrunken brains and a great deal of cracking of the sections, perfusion fixation using a Watson-Marlow pump at 2ml/min or 1ml/min resulted in little improvement as did perfusion fixation using gravity feed.

Upon recommendation a different fixative was used (D. Graham, Professor of Neuropathology, Glasgow University, Personal Communication); F.A.M. which is composed of one part 40% formaldehyde, one part glacial acetic acid and eight parts methanol. This fixative is faster and more appropriate for nissl staining than paraformaldehyde with gluteraldehyde but is not suitable for immunohistochemical techniques because the methanol and acetic acid destroy the antigenic properties of the tissue.

To reduce dark-cell artefact formation after fixation, the fixed pup was decapitated and the entire head placed in FAM fixative for a minimum of 4 hours. The brain was dissected out of the head and placed in fresh fixative and then left overnight. The improvement was already noticeable in that the brains were much firmer and easier to remove from the skull.

2.6.3 Processing

The water content of the FAM fixative is minimal and so for preparation for wax embedding the brains were washed three times for 30 minutes in 100% absolute alcohol. Then the brains were cleared in xylene as before. The heat of the wax was thought to be the source of the cracking problems and so molten paraplast wax was placed in a 60^{o}C incubator for 30 minutes before the brains were put into the wax. To ensure that all xylene was removed from the brains each brain was taken through 5 changes of wax and left for 30 minutes for each change. The brains were embedded after the fifth and final change of wax.

The use of plastic moulds led to the formation of a great many air bubbles around the embedded brains. Metal moulds were therefore used. The brains were placed into the wax mould and manoeuvred into an upright position using heated forceps. As soon as the top layer of wax had hardened the mould was slowly covered with cold water and then left for around 2 hours.

After sectioning on the microtome and staining it was possible to see a great improvement in the quality of the sections. The number of dark cell artefacts was negligible and the brains were no longer shrunken and mis-shapen.

2.6.4 Procedure for FAM Perfusion Fixation

This fixative was used for processing brains required for standard histochemistry. The neonatal rats were perfusion fixed at 4 hours, 3 days, 7 days, 14 days and 21 days after delivery by Caesarian section.

a) Procedure for FAM fixation of neonatal rats at 4 hours or 3 days of age

The rats at 4 hours old were anaesthetised using diethyl-ether and rats at pnd3 were anaesthetised using 10ml/kg 10% Sagatal (6mg/ml phenobarbital). When each rat was anaesthetised a blunted needle (23 gauge) was inserted into the left ventricle and the rats was perfusion fixed using a pulseless infusion pump (Carnegie Medicine, CMA 100) flowing at 1ml/min. 1ml of heparinised physiological saline was infused followed by 5ml of FAM for 4 hour old rats or 10ml of FAM for 4 day old rats. The FAM was at

room temperature. If the rat's neck was not rigid fixation was poor and so the histology experiment was terminated. When fixation was adequate the rat was decapitated and the head left in FAM for 4 hours. The brain was then dissected out of the skull and left overnight in fixative at 4°C.

b) Procedure for FAM fixation of rats at pnd7

Rats which were 7 days old were anesthetised using 10ml/kg 10% Sagatal and then perfusion fixed using a Masterflex (7014-20) pump (Cole Palmer Instrument Co.) at 5ml/min. The rats were perfused with 5-10ml of heparinised, physiological saline followed by 25-40ml of FAM at 37°C. The decapitated head was left in fixative for 4 hours and the brain was removed and left overnight in fixative at 4°C.

c) Procedure for FAM infusion of rats at pnd14 or pnd21

Rats at pnd14 or 21 were anaesthetised using 50mg/kg Sagatal (60mg/ml) and then perfusion fixed using a Masterflex pump at 10ml/minute. The rats were perfused with 15ml of saline followed by 50-100ml of FAM at 37°C. The rat was decapitated and the brain left in situ for 4 hours before being placed in fixative at 4°C and then left overnight.

2.6.5 Fixation Procedure for Immunohistochemical Staining

A parformaldehyde with gluteraldehyde fixative was used for the preparation of rat brains which were to be stained using immunohistochemical methods. The fixative was prepared by dissolving 4g of paraformaldehyde in 25ml 0.1M sodium phosphate buffer (pH 7.4) heated to 80°C. Any remaining traces of paraformaldehyde were removed by adding drops of 2M NaOH. The solution was made up to 4% paraformaldehyde by the addition of 75ml of buffer. 50µl of gluteraldehyde was added to 100ml of 4% paraformaldehyde (pH 7.4) to give a final concentration of 0.05% gluteraldehyde.

Rats were perfusion fixed at pnd 4, 7, 14 or 21. Anaesthesia was performed as described for FAM fixation. Rats at pnd 4 were perfused at 1ml/min with a flush of 1ml heparinised, physiological saline followed by 10ml fixative. Rats at pnd 7

were perfused at 5ml/min with a flush of 5ml heparinised saline followed by 50ml of fixative. Rats at pnd 14-21 were perfused at 10ml/min with a flush of 15ml heparinised saline followed by 75-100ml fixative.

Following perfusion the brain was removed and left in fixative for 30 minutes. The brain was then transferred to a post-fixative of 10% sucrose in 4% paraformaldehyde and left at $4^{o}C$ for a minimum of 24 hours before sectioning.

2.6.6 Fixation Procedure for Adult Rat Brain Tissue.

10% buffered formalin was used as a fixative for the preparation of adult rat brains for frozen sectioning using a cryostat. The fixative was prepared by adding 4g sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$), followed by 6.5g anhydrous disodium phosphate (Na_2HPO_4) to 1000ml of 10% formalin solution. The pH was corrected, if necessary, to pH 7.4.

The rats were anaesthetised with Euthatal (200mg/kg pentobarbital) and perfused with physiological saline and 150ml of the fixative using a pump (Second Nature Whisper 1000) flowing at 20-25 ml/min. After fixation the rats were decapitated, the brains removed and left in 10% buffered formalin until required.

2.6.7 Procedures for the Sectioning of Neonatal and Adult Rat Brains

a) Microtome

The brains from rats perfused at 4 hours to 7 days of age were embedded in molten paraffin wax. The wax-embedded brains age were sectioned on a microtome (Spenser 820). The cooled wax block was trimmed and firmly held against a heated brass-chuck. After the chuck had cooled it was locked into the microtome and ribbons of coronal sections at 6μm were cut from the block.

The ribbons of sections were floated out on distilled water at $55^{o}C$ and then placed onto albumen coated slides. The slides were stored overnight, in a vertical position, at $37^{o}C$ to dry the sections.

b) Cryostat

A Cryostat (Bright OTF/AS/MR/D) was used to take frozen sections from brains of rats fixed at pnd14 to adult. The neonatal rat brains which had been fixed in FAM were placed in 10% buffered formalin 24 hours before sectioning to promote the freezing process. The adult rat brains were washed for 60 seconds in distilled water before being frozen.

The front of the brain was trimmed slightly to provide a flat surface for mounting the brain. The brain was placed onto the chuck, using Tissue-tec (BDH Ltd, UK) as an adhesive, and frozen using Cryospray (BDH Ltd, UK).

Neonatal rat brains were placed in a cryostat at -18^{o}C and left for 10-15 minutes before coronal sections at 15μm were taken. Adult rat brains were placed in the cryostat at -27^{o}C, left for 15 minutes and then sectioned coronally at -20^{o}C (control and hypoxic rat brains) or horizontally (hippocampally-lesioned rat brains) at 30μm. All sections were placed onto chrome alum coated slides.

c) Freezing microtome

A freezing microtome was used to section rat brains which were to be stained immunohistochemically. The front part of the brain was trimmed slightly and the brain was placed onto a piece of filter paper on top of a chilled platform. The brain was frozen using dry ice and alcohol. 30-40μm sections were taken in the coronal plane and then floated into wells containing 50mM phosphate-buffered-saline (PBS).

2.6.8 Procedures for the Staining of Rat Brain Sections

(a) Cresyl fast violet (Bancroft and Stevens, 1977)

This Nissl stain is used to demonstrate cell distribution and cytoarchitecture and stains nuclei and Nissl a purplish-blue colour. Dead or dying pyknotic cells are stained a very dark purple. The stain was used for immature and adult rat brain sections.

The wax-embedded sections were left in xylene for 10 minutes to remove all of the wax. The frozen sections, on chrom alum slides, were dehydrated through 70%, 80%, 90% and 3 x 100% absolute alcohol and the slides were left in xylene for 40 seconds.

The following procedure was used for cresyl staining all sections:-

1) The sections were quickly rehydrated through the alcohols from 100% to 70% and then left in 70% alcohol + 0.5% glacial acetic acid for 5 minutes.
2) The sections were washed in distilled water, then placed in 0.1% Cresyl Violet (Sigma Chemicals Co) solution and left for 5-10 minutes.
3) The sections were washed in distilled water and the stain differentiated in 70% alcohol + 0.5% glacial acetic acid.
4) The sections were dehydrated and cleared in xylene. DPX mounting medium was used for mounting the sections and when the coverslip was in place the sections were left to dry overnight.

(b) Haemotoxylin and Eosin stain (Bancroft and Stevens, 1977)

The Mayers alum-haemotoxylin stain was used as a nuclear stain in conjunction with eosin which stained the cytoplasm and demonstrated the general cytoarchitecture of the sections. The haemotoxylin stained nuclei a red colour which was converted to a blue-black colour using the alkali lithium carbonate. The eosin stained the cytoplasm a pink colour. (Any dead and dying cells stained a very dark colour).

1) Preparation of Haematoxylin - Mayers solution

0.5g of haematoxylin (Gurrs Certistain) was dissolved in 0.5ml of absolute alcohol and then added to 500ml of distilled water which contained 25g of aluminium potassium sulphate. The solution was mixed well, and 0.1g of sodium iodate was added to the haematoxylin the solution mixed and left to stand overnight. 0.5g of citric acid and 25g of chloral hydrate were added. The solution was heated to boiling point, allowed to cool and the filtered. 200ml of the haemotoxylin solution was placed in a staining bath.

2) Preparation of Eosin

A 0.1% solution of eosin was prepared by dissolving 200mg of Eosin-Y (Sigma Chemical Co) in 200ml of distilled water. The solution was poured into a staining bath.

3) Procedure for haematoxylin and eosin staining

Waxembedded sections were de-waxed in xylene and frozen sections were dehydrated, as for Cresyl violet, and left in xylene for 40 seconds.

The following procedure was used to stain all sections:-

i) sections were rehydrated and then washed in distilled water.

ii) The sections were placed in haematoxylin for 15 minutes and then washed in distilled water.

iii) The red haematoxylin stain was converted to blue by washing the sections in a saturated solution of lithium carbonate.

iv) The stain was differentiated in 70% absolute alcohol + 5% HCl and then the sections were washed under running tap water for 15 minutes.

v) The sections were left in eosin for 15 minutes, washed in distilled water and then in distilled water containing 1% potassium alum for 30 seconds.

vi) The sections were dehydrated, cleared in xylene and DPX was used as the mounting medium.

(c) Glial fibrillary acidic protein (GFAP)

This immunohistochemical stain is used to demonstrate the presence of astrocytes following mechanical damage or trauma to the CNS. The staining procedure is based on the antigen-antibody reaction using rabbit antiGFAP antibody as the antibody and the avidin-peroxidase technique to label the antibody.

The following describes the procedure used for GFAP staining:-

1) The free-floating sections were washed 4 times using 50mM PBS (pH 7.4) containing 0.3% Triton-X and 0.001% sodium axide.

2) The sections were incubated in 2% goat serum (Scottish Antibody Production Unit) at room temperature for 1 hour with continuous shaking throughout.

3) The goat serum was removed and the sections were then incubated with rabbit antiGFAP (Dakopatts, Dako Ltd, UK). The antibody was diluted 1 in 2000 with 50mM PBS. 0.4ml antibody were added to each well and the sections were incubated overnight at $4^{o}C$.

4) The anti-GFAP was removed and the sections washed 2 times with 50mM PBS + 0.3% triton-X + 0.001% sodium azide.

5) The sections were incubated with a secondary biotinylated goat anti-rabbit antibody (1 in 2000 dilution). The sections were incubated for 45 minutes at room temperature.

6) The sections were washed twice with 50mM PBS and then incubated for 45 min with a avidin/peroxidase complex (Vectastain ABC kit, Vector Laboratories) which was prepared 30 minutes before the incubation.

7) The sections were washed once with 50mM PBS and then once with 0.1M Tris-buffer (pH 7.4).

8) A diaminobenzidine (DAB) solution was prepared using a commercial kit (Vector-Peroxidase Substrate Kit, Vector Laboratories Ltd, UK). DAB solution was added to each well and the sections left until the brown stain had developed which usually took 5 minutes.

9) The sections were washed 4 times with 50mM PBS and then floated onto gelatin coated slides. The sections were left to dry overnight.

10) The sections were dehydrated in 95% and 100% absolute alcohol, cleared in xylene and DPX was used as the mounting medium.

2.6.9 Procedure for Measurement of Hippocampal Areas in Adult Rat Brain Sections

As the adult rat brains were sectioned at 30 μm it was not possible to perform cell counts with any great degree of accuracy. Although cell counts were not possible it was believed that some attempt to measure cell density, even

if indirectly, should be made. The brain region most likely to be affected by an hypoxic-ischaemic episode was believed to be the hippocampus and so measurements were made of the width of the CA1 region of both control and hypoxic brains. The width of the dentate gyrus in the same section was also recorded as this region is not usually affected in moderate hypoxic-ischaemia and so changes here might reflect histological artefact.

The measurements were made through the use of an image analyser (Seescan) at the level of -4.8 ± 0.2 mm from Bregma (Paxinos and Watson, 1989). At a magnification of 250x three adjacent measurements of 300 μm segments along the length of the CA1 region could be made on the left and right side of each brain. The upper and lower blade of the dentate gyrus were also measured in each section.

All of the measurements were statistically compared using a two-way between subject ANOVA design (CLR-ANOVA programme) and significance was limited to $p < 0.05$.

2.7 DEVELOPMENT OF NEONATAL RATS : GROWTH CURVE AND BEHAVIOURAL ASSESSMENT

The development of two groups of neonatal rats was assessed. The first group, Hypoxic Rats, were rats which had been exposed to a 30 minute *in utero* occlusion followed by a 20 minute recovery period. The second group, Control rats, were the litter-mate controls for each of the hypoxic rats.

Following resuscitation, each rat was placed in an incubator, heated to 37^{o}C, and care was taken to keep the hypoxic rats separate from the control rats. The neonatal rats were monitored for a minimum of 2 hours during which time the rats were turned every 15 minutes. Any rat which was not breathing was manually stimulated until the

breathing reflex returned. At the end of the observation period a maximum of 6 rats were chosen for cross-fostering, where possible 3 hypoxic rats and 3 control rats were selected.

The tail of each rat was marked by an independent observer using water-proof indelible marker pens. The two groups were mixed together such that all further measurements and observations could be performed blind.

2.7.1 Procedure for the Measurement of the Growth Curve

Before being placed with the foster dam each pup was weighed and measured. The head-to-tail length was measured by placing thick surgical thread along the length of the rat from the tip of the nose to the tip of the tail and then measuring the thread against a ruler. These measurements were taken as the pnd0 size of each neonatal rat. After the weight and length had been recorded the neonatal rats were placed with the foster dam according to the method stated previously. The litter was checked 3-4 hours later to see if any rats had died and to replace any pen-marking removed by the dam.

The following morning between 9.30-10.30 a.m. the entire litter, including Cob rat pups, was removed from the dam and placed in an observation box. The tail identification marks were re-marked and the weight and length of each cross-fostered pup was recorded.

The weight and head-to-tail length of each cross-fostered neonatal rat was recorded daily from pnd0 to pnd23 then on pnd25, 28, 33, 36, 39 and on pnd42. Final measurements were recorded when the rats were around 3 months of age.

As the rat pups were growing a note was made of other developmental features, which were the ears lifting from the head, the day at which the eyes were fully open and the development of pigment and fur covering.

For the duration of the growth curve and behavioural assessments all of the litters were housed in the same room but in isolation from other rats. All assessments were performed within this room which was on a 14 hour light and a 10 hour dark cycle.

2.7.2 Procedures for Behavioural Observations from pnd0 to pnd42

a) Assessment from pnd0 - pnd11

No behavioural assessment was made on pnd0 although note was taken of any unusual activity such as convulsions. On pnd1 between 9.30 - 10.30a.m. the entire litter was removed from the dam and placed in a perspex observation box (40 x 50 x 30cm). The floor of the box was covered with a thin layer of wood-shavings. The area around the box was heated using an infra-red lamp to maintain neonatal rat body temperature while the litter was being assessed. The lamp was used until each rat had grown sufficient body-fur for independent thermo-regulation. The litter was left undisturbed in the box for an habituation period of 20 minutes. Following the measurement of the weight and length of each rat the litter was grouped together and placed near the heat source. One pup was removed and placed in another, identical, observation box. The pup was then observed for 3 minutes and any activity was recorded. The level of activity was scored according to the following 4-point scale:-

Score	Activity
0	No movement other than breathing for greater than 1.5 minutes
1	Very limited movement, for example, head turns and limb movement, with no change of body location for greater than 1.5 minutes
2	Some change of body location, for example crawling, but limited to less than 1.5 minutes
3	A lot of movement, many changes of body location for greater than 1.5 minutes

After the 3 minute observation period the righting-reflex of each rat was tested by placing the rat on its back on a flat level surface and recording the amount of time taken to right itself. A maximum of 2 minutes was allowed for the righting-reflex because by this point the rat had usually given up any attempt to right itself and also this time limit prevented excessive heat loss due to isolation from the litter.

Although the Cob Wistar pups were removed along with the cross-fostered rats they were not measured or assessed. Following completion of all the assessments the litter was placed back with the dam. The time away from the dam for each litter was restricted to a maximum of 50 minutes (20 minutes habituation + 30 minutes observation).

Behavioural assessments were made on alternate days from pnd1 to pnd11 and from pnd11 onwards different assessment techniques were used.

(b) Assessment from pnd11 to pnd42

On pnd11 the rats were, on average, five times heavier and two times longer than their original sizes. By this time most rats scored 3 in the activity score and so this method was no longer suitable for accurate activity assessments.

The observation box was divided into 4 equal sized quadrants of 20 x 25cm. At the start of the 3 minute observation period the rat was placed in the top left quadrant, the clock started and then as the rat moved around the box each quadrant cross was recorded.

Following the 3 minute activity assessment each rat was tested for the righting-reflex. The quadrant-crossing assessment began on pnd11 and was performed on alternate days until pnd21 when the rats were weaned. Previous studies have shown a decline in activity the day after weaning (Bolles and Woods, 1964) and so assessments were performed on pnd22 and pnd23. Further assessments were performed on pnd25, 28, 33, 39 and 42 by which time the rats were too big for quadrant-crossing to be measured with any accuracy.

After 42 days the rats were segregated according to sex and some litters were mixed. The Cob-Wistar males were taken away from the fostered Lister-hooded rats. A maximum number of 4 rats per cage was used and no rats were housed alone. At 3 months of age further testing of functional activity occurred but between 42 days and 3 months there was no testing. During this period the rats were 'handled' 2-3 times a week in order to maintain the association which had developed between the

experimenter and the rats. The handling procedure involved replacing the tail identification marks and physical contact with the rats in each cage for a period of not less than 3 minutes.

All developmental parameters and activity ratings were subjected to the unpaired t-test to detect any statistically significant differences between the control and hypoxic groups.

2.8 ASSESSMENT OF SPATIAL MEMORY IN CONTROL, HYPOXIC AND HIPPOCAMPALLY LESIONED ADULT RATS USING A WATER-MAZE

When the hypoxic rats and their littermate controls reached 3 months of age their spatial memory was assessed in an open field water maze (Morris, 1984). A group of 4 hippocampally lesioned rats was used as a positive control for the experiment (Morris *et al*, 1982).

2.8.1 Surgical Procedure for the Hippocampal Lesion

The rats used were male Lister-hooded rats which had been handled frequently during their development to control for any bias associated with the handling of the other two groups. The rats were anaesthetised using tribromoethanol (Avertin; 10ml/kg), and then placed on a Kopf stereotaxic instrument. The head was secured such that bregma and lambda were at the same level (nosebar - 3.3mm). A small area of bone was carefully removed to expose the brain above the region to be lesioned.

The lesions were performed bilaterally using ibotenic acid according to an adjusted method from Jarrad (1989). Ibotenic acid was dissolved in PBS (pH 7.4) at a concentration of 10μg/1μl and then drawn into a 2μl Hamilton syringe. In all there were 24 injection sites with 12 injections on each side and table 2.4 shows the coordinates for each injection site. Bregma was used as the zero reference point for the

A-P and M-L coordinates and the cortical surface was the reference point for the D-V coordinates. The volume of ibotenic acid injected at each site was 0.1µl except where the site is marked with an * where the amount was 0.05µl.

TABLE 2.4 STEREOTAXIC COORDINATES FOR THE HIPPOCAMPAL LESIONS
(Coordinates shown are in mm)

A-P	M-L	D-V
-2.9	1.0	-3.4
-3.5	1.4	-2.6*, -3.4*
-3.5	3.0	-3.0
-4.5	2.6	-2.3*, -3.3*
-4.5	3.7	-3.0
-5.3	3.9	-7.0
-6.1	4.1	-3.8
-6.1	5.1	-4.0, -4.9, -5.8

The syringe was slowly lowered to the point of injection and the ibotenic acid injected manually over a 2-5 minute period. The syringe was left in position for 5 minutes before moving to the next site. The entire procedure took between 2.5 to 3.0 hours during which time anaesthesia was maintained using Avertin. Following completion of all 24 injections the wound was sutured, the rat removed from the stereotaxic frame and then placed in a recovery box on top of a warming plate at 37^{o}C. When fully conscious, the rat was returned to the holding room and allowed a minimum of 8 full days recovery before testing began.

2.8.2 Place-Navigation in an Open-Field Water Maze

a) Apparatus

The open-field water maze was constructed according to the design of Morris (1984) and consisted of a glass fibre pool 2.0m in diameter and 0.6m high, filled with water to a depth of 0.3m. The water, at 25 ± 1°C, was replaced daily by an automated drainage and filling system which was controlled by a time switch. In order to conceal the platform the water was made opaque by the addition of powdered milk. The pool was housed in the centre of the testing room with a series of prominent visual cues around the room which included an unused section of primate cage, cupboards and posters on white walls.

The platform consisted of a length of sealed plexiglass tubing 10cm in diameter which had been painted white and filled with stones to weigh it down. The top of the platform was 1cm below the surface of the water and was visible from above but not along the surface of the water.

The rat's swimming behaviour was monitored by a video tracking system. A video camera was mounted above the centre of the pool such that the field of view included the entire pool surface. The camera was connected to a video recorder (Betamax stereo BNR, Sony) and to a tracking device (HVS Image Analysing Ltd, Kingston, UK). The room was brightly lit, using 4 x 500 Watt halogen flood lights, to improve the visual contrast between the black head of the Lister-hooded rats and the white surface of the pool. The tracking device was automated to track the head of the rats and the image was relayed to a computer-operated system (Archimedes) which sampled the X and Y co-ordinates of the rat's position at a frequency of 10Hz.

A programme called Water Maze ((c) R.G.M. Morris and R. Spooner) converted the X and Y co-ordinates into various objective measures including the swim path of the rat, the time taken to reach the platform (escape latency), the length of the swim path and the time spent in each quadrant of the pool.

The platform was placed in the centre of either the north-east or the south west quadrant of the pool. There were six start positions around the pool, north, east, south, west, south east and north west. Switches placed at the N, E, S and W positions around the outside of the pool were used to start the tracking system. One of three termination switches around the testing room or the adjoining equipment room was used to halt the tracking system at the end of each trial.

b) Testing Procedure - Acquisition

The different ages of the rats meant that the testing sessions had to be spread over 3 weeks to test 3 groups of rats which were all of a similar age. Group 1 included 5 control and 3 hypoxic rats, group 2 contained 6 control and 3 hypoxic rats and group 3 contained 3 control rats, 2 hypoxic rats and 4 hippocampally lesioned rats. The control and hypoxic rats were still anonymous but the lesioned rats were known to the experimenter.

On the Friday before the testing session began each rat was given a habituation session. This involved placing the rat in the pool (no platform was used) and allowing the rat to swim freely for 60 seconds. The rat was removed from the pool, dried and then returned to the group cage. This session allowed the rats to become accustomed to the water and to the testing room.

The following Monday testing proper began. The three testing groups were divided randomly such that half were assigned the north-east platform position and the other half were assigned the south-west platform position. The rats were given six trials a day for 4 days. Each trial lasted a maximum of 120 seconds and there was an inter-trial interval of 30 seconds.

At the start of each trial the rat was released from one of the six start positions, the tracking system started and the experimenter withdrew to the adjacent equipment room. The trial was terminated as soon as the rat climbed onto the platform. If the rat did not find the platform within the allowed 120 seconds it was placed onto the platform by the experimenter. During the 30 second intertrial interval the rat remained on the platform and if it jumped off the rat was placed back onto the platform.

After completion of the sixth trial the rat was dried and then returned to the group cage. The six start positions were randomly rearranged each day such that a different sequence of start positions was used for each of the four days.

c) Transfer Test

Over the 4 days of training the rats used the extra maze cues around the room to learn the position of the platform. On the fifth day the retention of the task was tested in a transfer test where the platform was removed from the pool and the rat allowed to swim freely for 60 seconds. The subsequent analysis of the path taken by the rat showed the spatial bias for the platform position which the rat had developed over the 4 days of training.

After the transfer test had been performed the rats were anaesthetised with euthatal (200mg/kg) and perfusion fixed with 10% buffered formalin. The frozen brains were sectioned and then stained with Cresyl fast violet (see histology section 2.6).

d) Statistical Analysis

The data from each testing group was combined and then organised into three groups for control rats, hypoxic rats and hippocampally lesioned rats. The escape latencies for all 24 trials were subjected to ANOVA and where appropriate post-hoc comparisons were made using Tukeys (HSD) test. Analysis of performance during the transfer test was based on the percentage time spent in each quadrant during the testing period. ANOVA was also applied to the transfer test data to detect any differences in the overall performance of each group of rats.

CHAPTER THREE

RESULTS

3. RESULTS

3.1 BRAIN TISSUE METABOLITE CHANGES FOLLOWING HYPOXIA

The levels of brain tissue metabolites were assessed at the end of each experimental procedure. The foetal rat brains were removed and de-proteinised at 0, 1, 60 and 180 minutes after severance of the umbilical cord. Following preparation of the tissue six metabolites were assessed; lactate, glucose, ATP, ADP, AMP and PCr. The changes in levels of each metabolite will be discussed separately.

3.1.1 Brain Tissue Lactate

Tables 3.1-3.4 summarise the mean concentrations of lactate in foetal rat brains after each experimental procedure. The changes in brain tissue lactate, following a hypoxic insult, at 0 minutes and 1 minute after delivery are illustrated in figures 3.1 and 3.2 respectively. When comparisons were made between the lactate content of the non-operated rat brains and the two sham-operated groups (Table 3.1) there were significant increases in lactate following exposure to anaesthetic ($p < 0.01$). As table 3.1 shows brain tissue lactate was lowest, at all measured time intervals in the non-operated rats which were delivered by Caesarian section without any previous exposure to anaesthetic. ANOVA of all the data from the non-operated and the sham groups did not expose a significant trend ($F(2,15) = 2.66$, $p = 0.10$) but a post-hoc pairwise comparison found a significant difference between the non-operated group and the sham-operated-without recovery group ($p < 0.01$). There was no significant difference between the two sham-operated groups or between the non-operated rats and the sham-operated-with recovery group.

The sham procedure involved exposure to 25 minutes of anaesthesia, and in order to check for any mechanical effect of the surgical procedure lactate levels from the two sham-operated groups were subjected to further comparisons with the lactate levels from the two 20-minute control groups using ANOVA (Tables 3.1 and 3.3). Inspection of the mean brain tissue lactate at zero minutes after recovery shows that the lactate concentration in both groups of control rats (Table 3.3) is slightly higher than

that in the sham-operated groups (Table 3.1) but these differences were not found to be significant ($p > 0.05$). ANOVA did, however, expose a significant overall effect of the recovery period ($F(1,22) = 6.30$, $p = 0.02$) which suggests that a 20 minute period without anaesthetic, after the surgical procedure, caused a reduction in brain tissue lactate back towards basal levels. This is in agreement with the results which show a significantly lower level of brain tissue lactate in non-operated rats (see above).

As tables 3.2 - 3.4 and figure 3.1 show, brain tissue lactate was consistently elevated in the hypoxic rat brains and reached a maximum of 22.29 ± 1.35 nmols/mg brain tissue in rats exposed to a 30 minute occlusion with a recovery period ("30 + 20", see Table 3.4). Pairwise comparisons, using the unpaired t-test, were made between each control and hypoxic group for all of the 3 different occlusion periods (10, 20 and 30 minutes, Tables 3.2, 3.3 and 3.4 respectively).

At 0 minutes after delivery there was a significant difference between hypoxic rats exposed to a 10 minute occlusion period and control rats ($p < 0.01$, without a recovery period and $p < 0.05$ with a recovery period, see Table 3.2). . Brain tissue lactate rose to higher levels in rats exposed to a 20 minute occlusion period and there was significantly more lactate in hypoxic rats in comparison with control rats ($p < 0.001$ without a recovery period and $p < 0.001$ with a recovery period, see Table 3.3). A 30 minute occlusion period was associated with even further increases in brain tissue lactate and that in hypoxic rat brains was significantly greater than that in control rats ($p < 0.001$, without and with a recovery period, see Table 3.4).

The ANOVA of all the data from control and hypoxic groups and for each occlusion period at 0 minutes after delivery exposed a significant effect of group ($F(1,76) = 186.41$, $p < 0.001$) which refers to a consistent difference between control and hypoxic rats whatever the length of the occlusion period. There was also a significant interaction between the length of the occlusion period and the groups ($F(2,76) = 11.77$ and $p < 0.001$) which was investigated further using post-hoc comparisons. The increase in lactate in the three different control groups was not found to be significant but significant differences were found between 10 minutes of

hypoxia and 30 minutes of hypoxia and also between 20 minutes of hypoxia and 30 minutes of hypoxia. These results would suggest that a 30 minute occlusion period produced the greatest increase in lactate in hypoxic rat brains.

A significant interaction was also exposed between the recovery period and the groups ($F(1,76) = 6.37$, $p = 0.01$) which was found to be due to a significantly higher level of brain tissue lactate in hypoxic rats exposed to a recovery period than in those exposed to hypoxia alone. The recovery period did not, however, have a significant effect on brain tissue lactate in control rats. After a 20 minute occlusion period, and immediately after delivery, there was a 64.0% increase in brain tissue lactate in hypoxic rats but if the occlusion was followed by a recovery period there was a significantly higher increase of 170.3% ($p < 0.02$, unpaired t-test). Similarly after a 30 minute occlusion period the mean percentage increase in lactate was significantly greater ($p < 0.02$, unpaired t-test) with a recovery period, 256.6% than without a recovery period, 147.7%. No such difference existed for rats exposed to only 10 minutes of hypoxia, which would suggest that not only did the longer occlusion periods produce the greatest increases in brain tissue lactate they also caused an even further increase during the recovery period rather than the decrease expected from the sham data.

Brain tissue lactate was assayed at one minute after delivery since this measurement could be more accurately controlled than the zero minute collection point. The majority of differences in brain tissue lactate prevalent immediately after delivery were still evident at one minute after delivery (see Tables 3.1 - 3.4) and those differences are illustrated in figure 3.2. Brain tissue lactate remained elevated in hypoxic rat brains in comparison with control rat brains and when comparisons were made, using the unpaired t-test, these differences were significant for 10 minute (Table 3.2), 20 minute (Table 3.3) and 30 minute (Table 3.4) occlusion periods. The ANOVA of all of the data collected one minute after delivery supported the significant differences between the control and the hypoxic rats ($F(1,74) = 148.77$, $p < 0.001$).

As with the data collected zero minutes after delivery, when lactate levels measured at one minute after delivery were subjected to analysis, there was a significant interaction between the length of the occlusion period and group ($F(2,74)$ 7.36, $p = 0.001$). When post-hoc comparisons were made, significant differences were found between 10 and 30 minutes of hypoxia and also between 20 and 30 minutes of hypoxia but no significant differences were found between the three control groups. This, in addition to the data at zero minutes after delivery, supports the observation that a 30 minute occlusion period produced the greatest accumulation of lactate in hypoxic rat brains. When ANOVA was performed no significant effect of the recovery period on control or hypoxic rat brain tissue lactate was found ($F(1,74) = 0.31$, $p = 0.58$). This observation means that any further increase in hypoxic brain tissue lactate during the recovery period may be significant at zero minutes after delivery but not at one minute after delivery.

It can be seen from inspection of tables 3.1 - 3.4 that the level of brain tissue lactate declined in all rats during the early post-natal period whether or not they had been exposed to hypoxia. Due to the difficulty in achieving long-term survival of those foetal rats delivered immediately after completion of the occlusion analysis of post-natal measurements was only performed on those rats which were exposed to an additional recovery period. The brain tissue lactate measured at 0, 1, 60 and 180 minutes after delivery in the non-operated and the sham-operated - with recovery groups (Table 3.1) was analysed using ANOVA. The time after delivery was found to have a significant effect on both groups of rats ($F(3,40) = 9.35$, $p < 0.001$) and when post-hoc comparisons were performed the level of brain tissue lactate at 180 minutes after delivery was significantly lower than at all of the other measured times after delivery. There were, however, no significant differences between zero and one minute and between zero and 60 minutes after delivery.

When data were available unpaired t-tests were performed between control and hypoxic groups at 60 and 180 minutes after delivery (results are shown in Tables 3.2, 3.3 and 3.4 for 10, 20 and 30 minute occlusion periods respectively) but no significant

differences were observed between the control and hypoxic rats. The ANOVA of all data for control and hypoxic rat brain tissue lactate (Tables 3.2 - 3.4) exposed a significant effect of time after delivery ($F(3,126) = 28.90$, $p < 0.001$). The level of lactate at 60 and 180 minutes after delivery was found to be significantly lower than that at zero or one minute after delivery. There was also a significant interaction between time and group ($F(3,126) = 12.84$, $p < 0.001$) which reflected not only the differences between control and hypoxic rat brain tissue lactate but also a difference in the rate of decline in post-natal lactate between control and hypoxic rats. When post-hoc comparisons were made there was no significant change in lactate between zero and one minute after delivery for control or hypoxic rats. For hypoxic neonatal rats there was a significant reduction in brain tissue lactate between zero and 60 minutes but not between 60 and 180 minutes after delivery. For control neonatal rats, as with non-operated rats, there was not a significant reduction in lactate between zero and 60 minutes but the decline between 60 and 180 minutes after delivery was significant. There was no significant interaction between time after delivery and the occlusion period ($F(6,126) = 0.92$, $p = 0.48$) which means that, although the longer occlusion periods caused a greater accumulation of brain tissue lactate, the duration of the occlusion period did not influence the rate at which lactate declined during the first 3 hours of post-natal life. These results suggest that following delivery by Caesarian section brain tissue lactate declines steadily over the first 3 hours of post-natal life. If, however, the foetal rat is exposed to an *in utero* hypoxic insult brain tissue lactate accumulates during the insult and then falls rapidly, to levels not dissimilar from control rats, in the first 60 minutes of post-natal life.

TABLE 3.1 BRAIN TISSUE LACTATE (nmols/mg brain tissue) - NON-OPERATED AND SHAM OPERATED RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	MEAN BRAIN TISSUE LACTATE	NO. OF RATS
NON-OPERATED	0	3.92 ± 0.22	6
	1	5.28 ± 0.45	6
	60	2.97 ± 0.40	6
	180	2.14 ± 0.30	6
SHAM OPERATED (without recovery)	0	5.79 ± 0.50	6
	1	7.02 ± 0.15	6
	60		
	180		
SHAM OPERATED (with 20 mins recovery)	0	4.79 ± 0.83	6
	1	6.85 ± 1.11	6
	60	5.53 ± 1.43	6
	180	2.08 ± 0.63	6

The table summarises the data (mean ± s.e.m.) for brain tissue lactate from normal (non-operated) rats delivered by Caesarian section, for sham operated rats exposed to 25 minutes of anaesthesia and sham operated rats exposed to anaesthesia + a 20 minute recovery period.
The unpaired t-test was used for comparison of the two sham-operated groups but there were no significant differences.

TABLE 3.2 BRAIN TISSUE LACTATE (nmols/mg brain tissue) : A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 10 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Lactate	No. of Rats	Mean Brain Tissue Lactate	No. of Rats	
10 MINS +	0	4.62 ± 0.59	6	10.53 ± 1.32	6	< 0.01
0 MINS	1	6.87 ± 0.66	6	13.02 ± 2.77	6	n.s.
RECOVERY	60	10.83 ± 2.27	5	7.84 ± 1.25	5	n.s.
	180	2.39 ± 0.31	2	2.10 ± 0.54	3	n.a.
10 MINS +	0	5.40 ± 0.75	6	12.94 ± 2.79	6	< 0.05
20 MINS	1	7.72 ± 1.60	6	14.06 ± 1.04	6	< 0.01
RECOVERY	60	7.92 ± 1.13	6	8.10 ± 1.35	6	n.s.
	180	1.73 ± 0.18	6	3.00 ± 0.63	6	n.s.

(n.s. - not significant, n.a. = not appropriate)

The table summarises data (mean ± s.e.m.) from hypoxic foetal rat brains and their littermate controls after a 10 minute occlusion period (top portion) and after a 10 minute occlusion followed by a 20 minute recovery period (bottom portion).

TABLE 3.3 BRAIN TISSUE LACTATE (nmols/mg brain tissue) : A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 20 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Lactate	No. of Rats	Mean Brain Tissue Lactate	No. of Rats	
20 MINS +	0	7.74 ± 0.70	7	12.69 ± 0.72	7	< 0.001
0 MINS	1	7.60 ± 0.99	7	15.23 ± 0.46	7	< 0.001
RECOVERY	60					
	180					
20 MINS +	0	5.49 ± 0.50	7	14.84 ± 1.45	7	< 0.001
20 MINS	1	7.80 ± 0.64	6	14.75 ± 1.84	6	< 0.01
RECOVERY	60	8.72 ± 1.57	5	10.35 ± 1.07	5	n.s.
	180	2.65 ± 1.00	4	5.66 ± 2.15	4	n.s.

(n.s. = not significant)

The table represents a summary of data (mean ± s.e.m.) collected from foetal rat brains exposed to a 20 minute occlusion and their littermate controls.

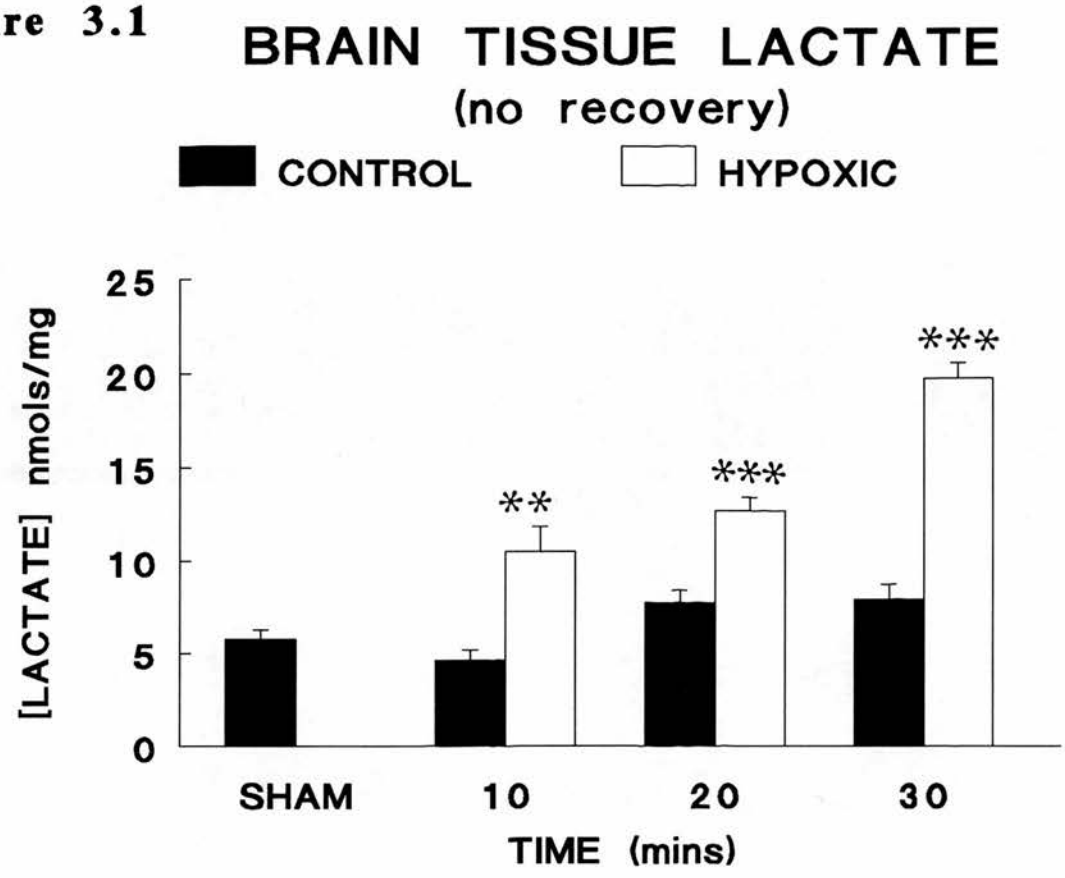
TABLE 3.4 BRAIN TISSUE LACTATE (nmols/mg brain tissue) : A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 30 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Lactate	No. of Rats	Mean Brain Tissue Lactate	No. of Rats	
30 MINS +	0	7.98 ± 0.82	7	19.77 ± 0.83	7	< 0.001
0 MINS	1	6.67 ± 0.76	7	17.45 ± 1.81	7	< 0.001
RECOVERY	60					
	180					
30 MINS +	0	6.25 ± 0.50	11	22.29 ± 1.35	11	< 0.001
20 MINS	1	7.28 ± 0.65	11	21.22 ± 0.50	11	< 0.001
RECOVERY	60	8.44 ± 1.35	6	11.03 ± 1.27	6	n.s.
	180					

(n.s. = not significant)

The top portion of the table summarises data (mean ± s.e.m.) collected from hypoxic rat brains after a 30 minute occlusion and from their littermate controls. The bottom half represents data from rats exposed to a 30 minute occlusion + a 20 minute recovery period.

Figure 3.1


BRAIN TISSUE LACTATE
(no recovery)
CONTROL
HYPOXIC
[LACTATE] nmols/mg
25
20
15
10
5
0
**
***
***
SHAM
10
20
30
TIME (mins)

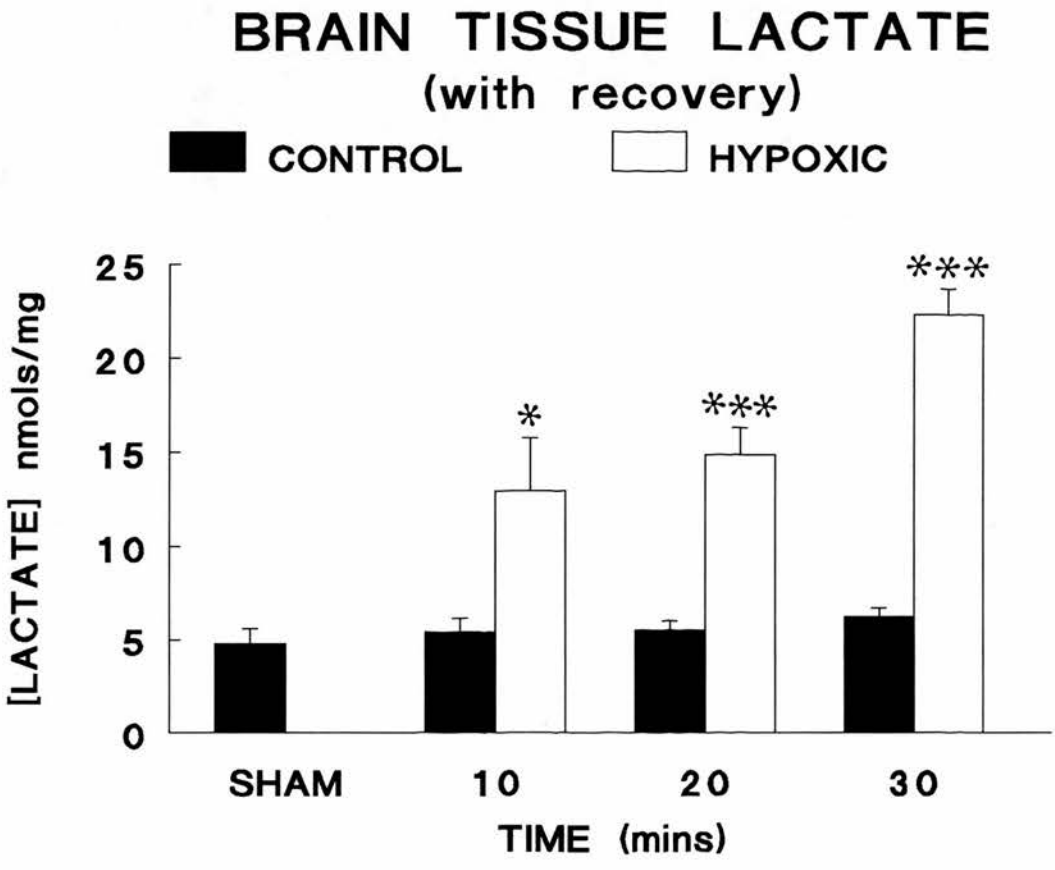
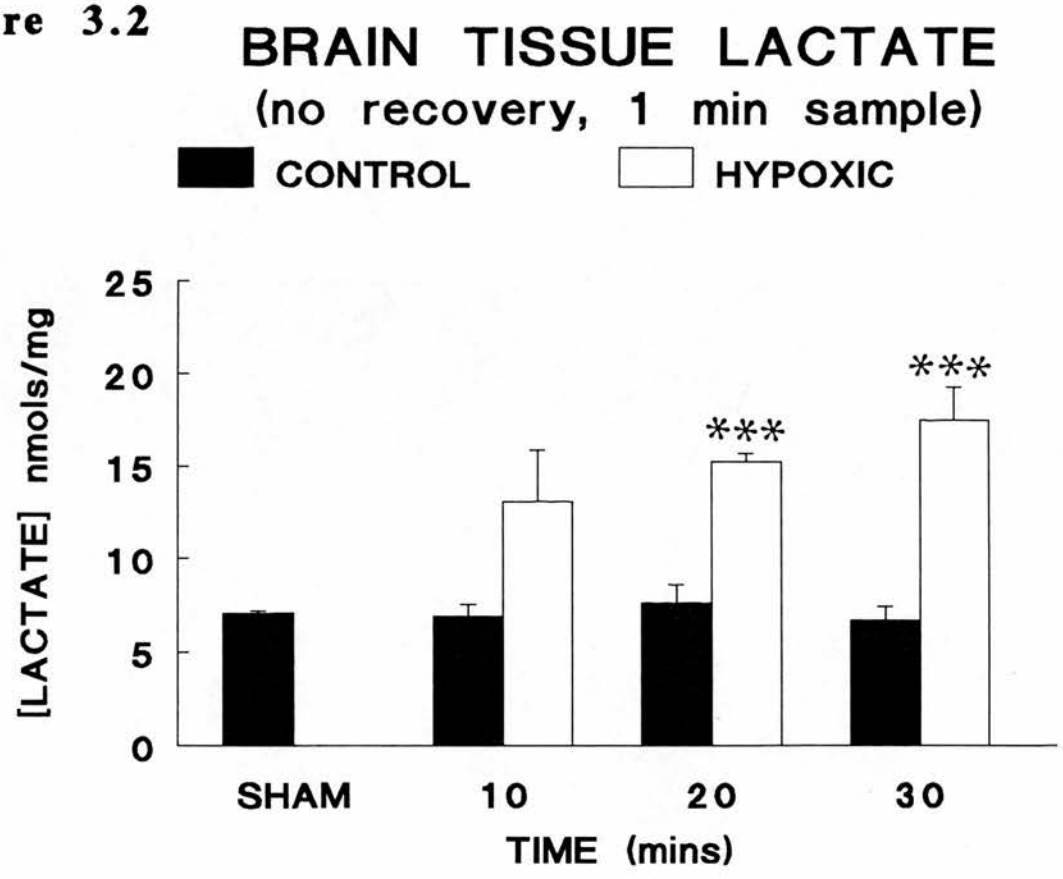

BRAIN TISSUE LACTATE
(with recovery)
CONTROL
HYPOXIC
[LACTATE] nmols/mg
25
20
15
10
5
0
*
***
***
SHAM
10
20
30
TIME (mins)

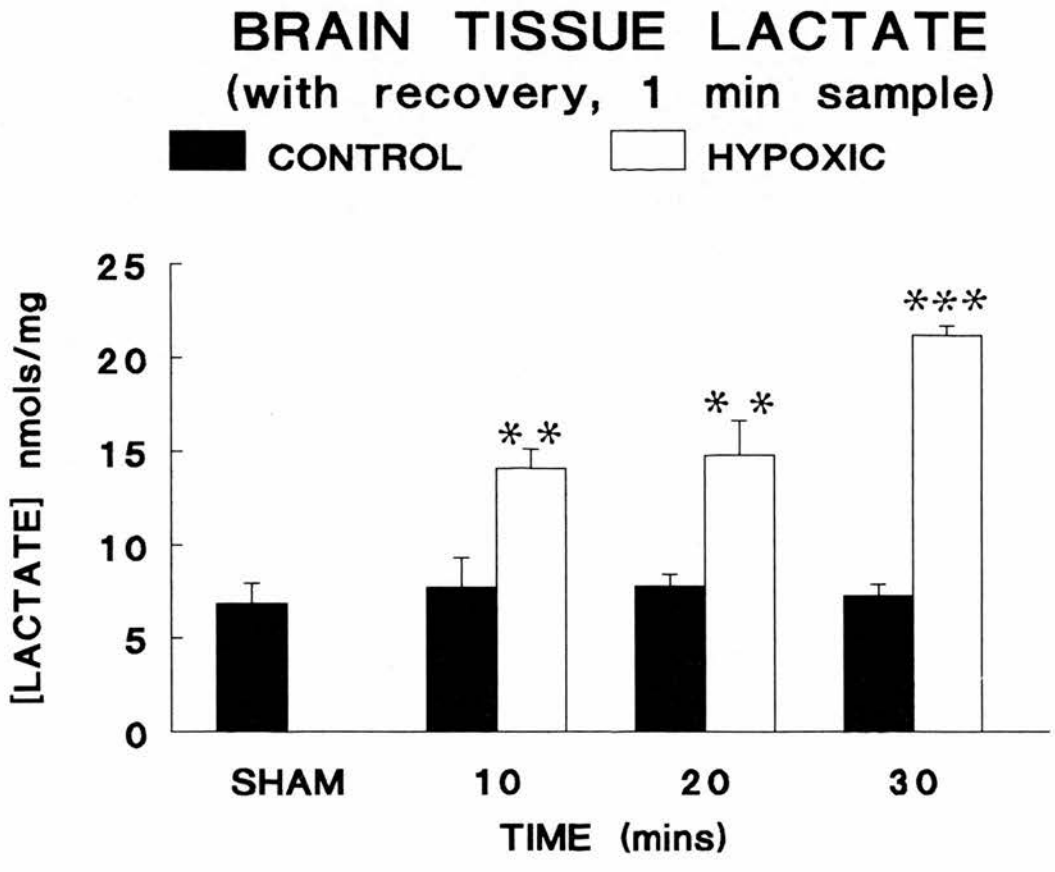
FIGURE 3.2 THE CHANGES IN BRAIN TISSUE LACTATE IN NEAR-TERM FOETAL RATS FOLLOWING IN UTERO HYPOXIA - 1 MINUTE AFTER DELIVERY

The two histograms represent the levels of brain tissue lactate (nmols/mg brain tissue), expressed as mean ± s.e.m., 1 minute after delivery by Caesarian section. The time refers to the length of the occlusion period.

See Figure 3.1 for details of the procedures. Statistical analysis was performed using the unpaired t-test. ** = $P < 0.01$, *** = $P < 0.005$.

Figure 3.2


BRAIN TISSUE LACTATE
(no recovery, 1 min sample)
CONTROL
HYPOXIC
[LACTATE] nmols/mg
25
20
15
10
5
0
***
***
SHAM
10
20
30
TIME (mins)


BRAIN TISSUE LACTATE
(with recovery, 1 min sample)
CONTROL
HYPOXIC
[LACTATE] nmols/mg
25
20
15
10
5
0
**
**
***
SHAM
10
20
30
TIME (mins)

3.1.2 Brain Tissue Glucose

A summary of the data obtained for all of the experimental groups can be found in tables 3.5 - 3.8 and figures 3.3 and 3.4 illustrate the changes in brain tissue glucose in neonatal rats immediately and one minute after delivery by Caesarian section.

Table 3.5 shows the level of brain tissue glucose in non-operated neonatal rats and in the two sham-operated groups. At all measured time intervals the level of brain tissue glucose in non-operated rats is lower than in sham-operated rats. ANOVA exposed a significant increase in brain tissue glucose following exposure to anaesthetic (non-operated versus sham-operated $F(1,10) = 44.30$, $p < 0.001$). There was, however, no significant difference between the sham-operated-without recovery and the sham-operated with recovery groups ($F(1,10) = 1.19$, $p = 0.30$). When comparisons were made between the sham-operated groups (Table 3.5) and the two 20 minute control groups (Table 3.7), there were no significant differences ($F(1,22) = 0.55$, $p = 0.47$) which suggests that the surgical procedure did not directly influence brain tissue glucose levels. There was a trend for the glucose levels with a recovery period to be higher than those without, but this was not significant ($F(1,22) = 3.23$, $p = 0.09$).

Tables 3.6 - 3.8 and figure 3.3 show that brain tissue glucose is consistently reduced following a hypoxic insult when measured immediately after delivery. When pairwise comparisons were performed, using the unpaired t-test, there was a significant reduction of 73.1% in brain tissue glucose following a 10 minute occlusion period ($p < 0.01$ - Table 3.6) but the reduction of 24.3% was not significant when the occlusion was followed by a recovery period. Following a 20 minute occlusion period (Table 3.7) there was a significant 63.9% reduction in glucose ($p < 0.02$) but with an additional recovery period the reduction in brain glucose of 19.1% was no longer significant. After a 30 minute occlusion period (Table 3.8) there was also a significant reduction of 78.3% in glucose ($p < 0.01$) and with the additional recovery period the reduction in glucose of 58.5% was still significant ($p < 0.01$).

The ANOVA of all data measured at zero minutes after delivery confirmed a significant trend for brain tissue glucose to be lower in hypoxic rats in comparison with control rats ($F(1,74) = 25.44$, $p < 0.001$). A significant effect of the recovery period was also exposed ($F(1,74) = 18.426$, $p < 0.001$) but the lack of a significant group - recovery period interaction ($F(1,74) = 0.69$, $p = 0.41$) suggests that the small but significant increase in brain tissue glucose during the recovery period was similar for both control and hypoxic rats. There was no significant interaction between the duration of the occlusion period and the group ($F(2,74) = 2.30$, $p = 0.11$) which suggests that extent to which brain tissue glucose was reduced was not significantly related to the length of the hypoxic insult.

The measurement of brain tissue glucose at one minute after delivery produced marginally different results to those observed at zero minutes after delivery (Tables 3.6 - 3.8 and figure 3.4). Following a 10 minute occlusion period (see Table 3.6) brain tissue glucose levels were reduced by 50.4% but this was not significant ($p > 0.05$, unpaired t-test). A recovery period after the 10 minute occlusion led to a slight, non-significant rise in glucose above control levels (14.5%) at one minute after delivery. Similarly, after a 20 minute occlusion period (Table 3.7) hypoxic brain tissue glucose was significantly reduced by 68.3% ($p < 0.01$) but with an additional recovery period glucose was marginally elevated by 73.3% above control levels ($p > 0.05$). A 30 minute occlusion period (Table 3.8) was also associated with a significant reduction of 61.2% in glucose ($p < 0.05$) whereas with a recovery period glucose was not significantly different compared with control levels.

Analysis of data collected at one minute after delivery using ANOVA found no overall difference between control and hypoxic rats ($F(1,72) = 1.50$, $p = 0.23$). There was, however, a significant effect of the recovery period ($F(1,72) = 14.89$, $p < 0.001$) which reflected the fact that the increase in brain tissue glucose during the recovery period was more marked at one minute after delivery. Although there was no significant interaction between group and recovery period ($F(1,72) = 3.31$, $p = 0.07$) the influence of the recovery period on the hypoxic rats was found to be particularly

significant ($F(1,72) = 16.78, p < 0.001$). This level of significance probably reflects the marginal increase in hypoxic brain glucose above control levels seen with the 10 and 20 minute occlusion + recovery groups (Tables 3.6 and 3.7).

The levels of brain tissue glucose at 0, 1, 60 and 180 minutes after delivery (see Tables 3.5 - 3.8) changed considerably and the pattern of change in post-natal glucose was dependent upon which surgical procedure the rats had been exposed to. The level of glucose in both non-operated and sham-operated (with recovery) rats (see Table 3.5) decreased between zero minutes and 60 minutes and then increased between 60 minutes and 180 minutes but, when analysed with ANOVA, this general trend was not found to be significant ($F(3,40) = 2.75, p = 0.06$).

ANOVA of all control and hypoxic rat brain tissue glucose data during the early post-natal period (Tables 3.6 - 3.8) found a significant effect of time after delivery ($F(3,126) = 4.60, p = 0.004$). There was no significant interaction between time and group ($F(3,126) = 1.28, p = 0.29$) which means that the changes in post-natal brain tissue glucose were similar for control and hypoxic rats. Post-hoc comparisons showed there was a significant increase in brain tissue glucose between zero and 60 minutes after delivery but the changes between 60 and 180 minutes after delivery were not significant. The rise in glucose between zero and 60 minutes in both control and hypoxic rat brains conflicts with the decrease observed in non-operated rats. As glucose is significantly higher in all rats exposed to anaesthetic the differing post-natal alterations in brain tissue glucose may reflect lingering effects of the anaesthetic.

In general the level of brain tissue glucose fell immediately in response to a hypoxic insult but within a 20 minute recovery period glucose levels increased in hypoxic rat brains and at one minute after delivery, whatever the length of the occlusion period, there were no significant differences between control and hypoxic rats. The anaesthetic might appear to have a more persistent effect on brain tissue glucose than an episode of intrauterine hypoxia.

TABLE 3.5 BRAIN TISSUE GLUCOSE (nmols/mg brain tissue) - SHAM OPERATED RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	MEAN BRAIN TISSUE GLUCOSE	NO. OF RATS
NON-OPERATED	0	0.73 ± 0.12	6
	1	0.62 ± 0.13	6
	60	0.31 ± 0.08	6
	180	0.74 ± 0.13	6
SHAM	0	1.98 ± 0.15	6
OPERATED	1	1.79 ± 0.27	6
(without recovery)	60		
	180		
SHAM	0	2.75 ± 0.69	6
OPERATED	1	2.19 ± 0.58	6
(with 20 mins	60	1.31 ± 0.27	6
recovery)	180	1.58 ± 0.54	6

The table summarises the levels of brain tissue glucose (mean ± s.e.m.) for normal rats (non-operated) delivered by Caesarian section, for sham operated rats exposed to anaesthesia and for sham operated rats exposed to a 20 minute recovery period. The unpaired t-test found no significant difference between the two sham-operated groups.

TABLE 3.6 BRAIN TISSUE GLUCOSE (nmols / mg brain tissue) : HYPOXIC RATS EXPOSED TO A 10 MINUTE OCCLUSION IN COMPARISON WITH CONTROL RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Glucose	No. of Rats	Mean Brain Tissue Glucose	No. of Rats	
10 MINS +	0	1.93 ± 0.38	6	0.52 ± 0.08	6	< 0.01
0 MINS	1	1.19 ± 0.31	6	0.59 ± 0.11	6	n.s.
RECOVERY	60	5.57 ± 0.98	5	5.89 ± 0.94	5	n.s.
	180	0.93 ± 0.02	2	0.74 ± 0.22	3	n.a.
10 MINS +	0	2.51 ± 0.38	6	1.90 ± 0.64	6	n.s.
20 MINS	1	2.49 ± 0.59	6	2.85 ± 0.49	6	n.s.
RECOVERY	60	4.23 ± 0.88	6	3.66 ± 1.16	6	n.s.
	180	1.20 ± 0.28	6	1.46 ± 0.31	6	n.s.

(n.s. = not significant, n.a. = not applicable)

The table shows a summary of data (mean ± s.e.m.) collected from rats exposed to a 10 minute occlusion and their littermate controls. The bottom portion represents those animals which were given an additional 20 minute recovery period.

TABLE 3.7 BRAIN TISSUE GLUCOSE (nmols / mg brain tissue) : HYPOXIC RATS EXPOSED TO A 20 MINUTE OCCLUSION IN COMPARISON WITH CONTROL RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Glucose	No. of Rats	Mean Brain Tissue Glucose	No. of Rats	
20 MINS +	0	2.16 ± 0.47	7	0.78 ± 0.18	7	< 0.02
0 MINS	1	2.05 ± 0.37	7	0.65 ± 0.09	7	< 0.01
RECOVERY	60					
	180					
20 MINS +	0	3.40 ± 0.69	7	2.75 ± 0.47	7	n.s.
20 MINS	1	2.47 ± 0.24	6	4.28 ± 1.21	6	n.s.
RECOVERY	60	3.32 ± 0.42	5	5.69 ± 1.27	5	n.s.
	180	3.35 ± 1.57	4	4.43 ± 1.73	4	n.s.

(n.s. = not significant)

The data shown (mean ± s.e.m.) were taken from rats exposed to a 20 minute occlusion and their littermate controls. Data from those rats which were exposed to an additional 20 minute recovery period are in the lower half of the table.

TABLE 3.8 BRAIN TISSUE GLUCOSE (nmols / mg brain tissue) : HYPOXIC RATS EXPOSED TO A 30 MINUTE OCCLUSION IN COMPARISON WITH CONTROL RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue Glucose	No. of Rats	Mean Brain Tissue Glucose	No. of Rats	
30 MINS +	0	2.90 ± 0.53	6	0.63 ± 0.19	6	< 0.01
0 MINS	1	2.60 ± 0.37	6	1.01 ± 0.48	6	< 0.05
RECOVERY	60					
	180					
30 MINS +	0	4.05 ± 0.53	11	1.68 ± 0.46	11	< 0.01
20 MINS	1	3.23 ± 0.75	11	1.82 ± 0.83	11	n.s.
RECOVERY	60	4.81 ± 1.39	6	6.04 ± 0.92	6	n.s.
	180					

(n.s. = not significant)

The table summarises data (mean ± s.e.m.) collected from foetal rat brains after a 30 minute occlusion and their littermate controls. Data from rats exposed to the additional 20 minute recovery period are in the lower half of the table.

FIGURE 3.3 THE CHANGES IN FOETAL RAT BRAIN TISSUE GLUCOSE FOLLOWING AN IN UTERO HYPOXIC EPISODE

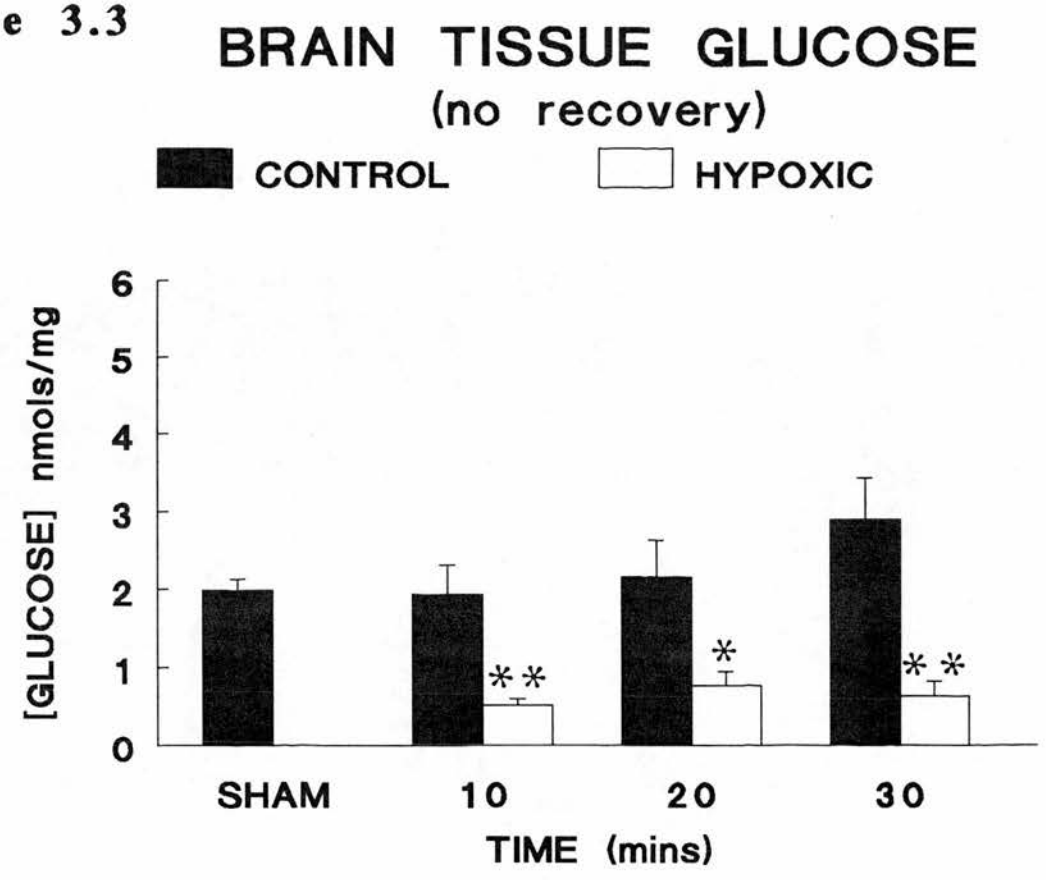
The histograms illustrate the mean levels of brain tissue glucose in rats immediately after delivery by caesarian section. The concentration of glucose is expressed as nmols/mg brain tissue. The time refers to the duration of the occlusion period.

The sham operated animals were anaesthetised for 25 minutes and a mid-line abdominal incision performed but no uteroplacental vessels were occluded. The bottom graph represents those rats which were exposed to a period of 20 minutes without anaesthetic after completion of the occlusion and the removal of the clips.

Following completion of the experimental procedure the brains were removed and homogenised in $HClO_4$ at $-10^{o}C$. The deproteinised tissue samples were assayed fluorimetrically according to the method of Lowry and Passoneau (1972). The concentration of brain tissue glucose was proportional to the change in fluorescence.

The data were subjected to statistical analysis using the unpaired t-test, * = $P < 0.05$, ** = $P < 0.01$.

Figure 3.3


BRAIN TISSUE GLUCOSE
(no recovery)
CONTROL
HYPOXIC
[GLUCOSE] nmols/mg
0
1
2
3
4
5
6
**
*
**
SHAM
10
20
30
TIME (mins)

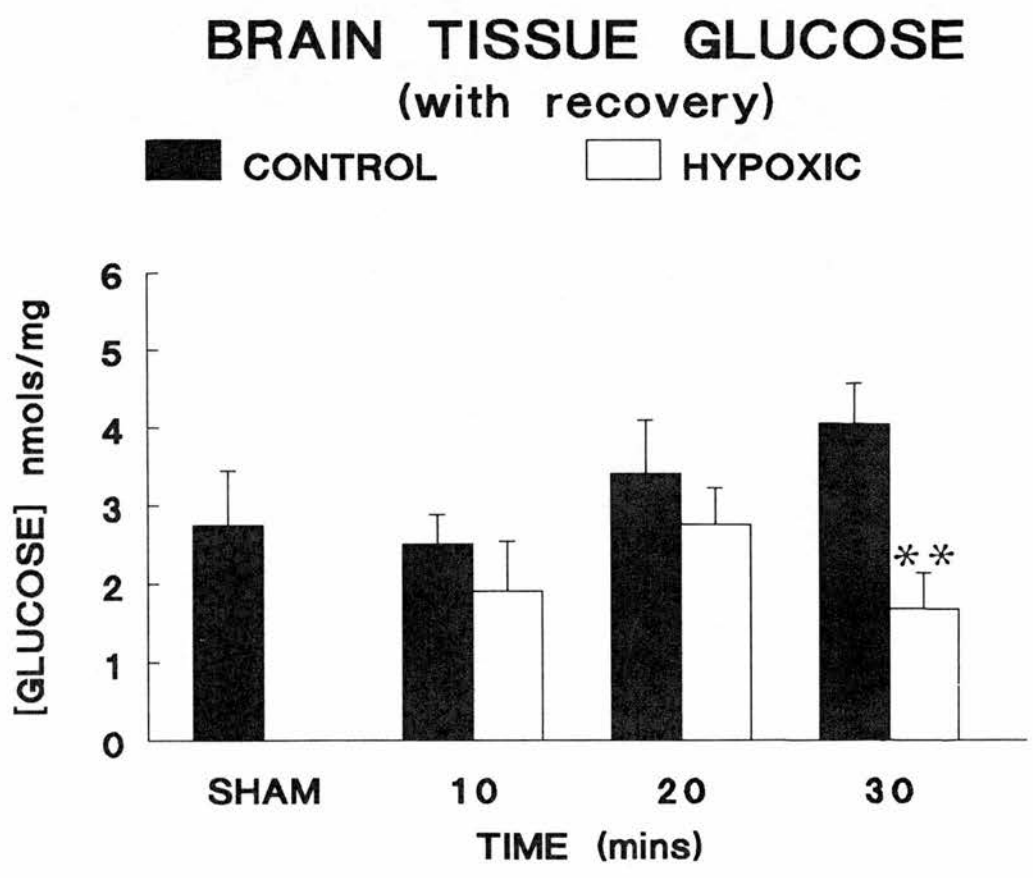
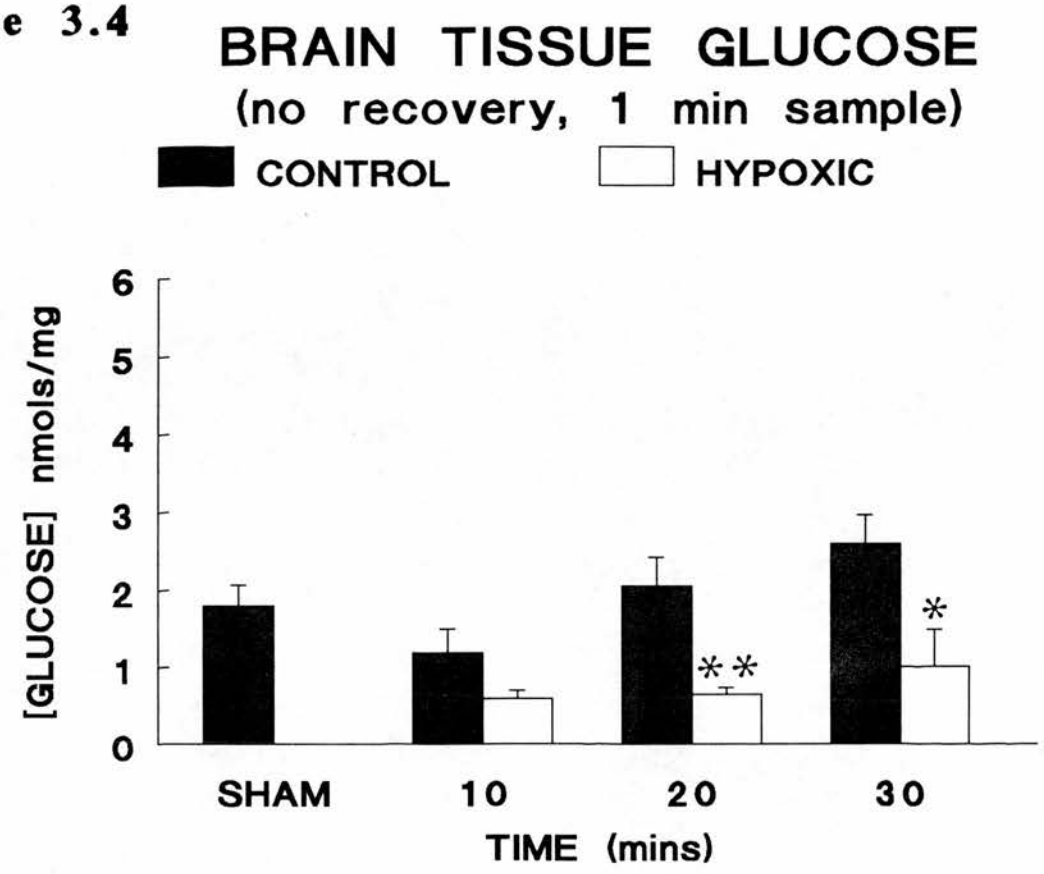

BRAIN TISSUE GLUCOSE
(with recovery)
CONTROL
HYPOXIC
[GLUCOSE] nmols/mg
0
1
2
3
4
5
6
**
SHAM
10
20
30
TIME (mins)

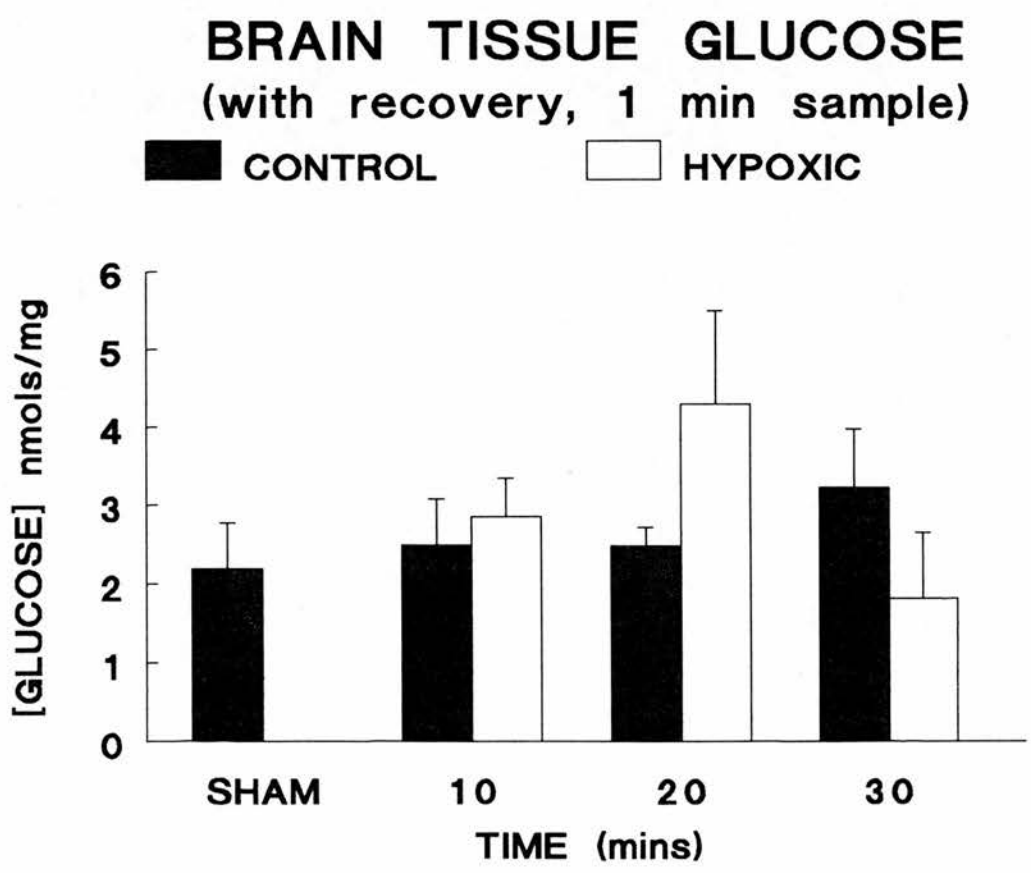
FIGURE 3.4 THE CHANGES IN BRAIN TISSUE GLUCOSE FOLLOWING AN IN UTERO HYPOXIC EPISODE ONE MINUTE AFTER DELIVERY BY CAESARIAN SECTION

The two histograms represent the concentrations of glucose (nmols/mg brain tissue) found in neonatal rat brains one minute after delivery. Time refers to the duration of the occlusion period. The bottom graph represents those data from rats exposed to an additional 20 minute recovery period. Refer to figure 3.3 for a description of procedures.

The data for control and hypoxic animals were subjected to the unpaired t-test to detect statistically significant differences. * = $P < 0.05$, ** = $P < 0.01$.

Figure 3.4


BRAIN TISSUE GLUCOSE
(no recovery, 1 min sample)
CONTROL
HYPOXIC
[GLUCOSE] nmols/mg
0
1
2
3
4
5
6
**
*
SHAM
10
20
30
TIME (mins)


BRAIN TISSUE GLUCOSE
(with recovery, 1 min sample)
CONTROL
HYPOXIC
[GLUCOSE] nmols/mg
0
1
2
3
4
5
6
SHAM
10
20
30
TIME (mins)

3.1.3 Brain Tissue ATP

Figures 3.5 and 3.6 illustrate the changes in control and hypoxic rat brain tissue ATP at zero and one minutes after delivery respectively and tables 3.9 - 3.12 summarise the concentrations of brain tissue ATP measured after each experimental procedure.

The ANOVA of brain tissue ATP observed in the non-operated and sham-operated groups (Table 3.9) identified a significant increase between the non-operated and sham-operated-with recovery groups ($F(2,15) = 6.17$, $p = 0.01$). However, there was no significant difference between the non-operated and sham-operated-without recovery groups which implies that the effect of anaesthesia on brain tissue ATP was only evident after a 20 minute recovery period. Although the level of brain tissue ATP in the sham-operated-with recovery group was greater than in the sham-operated-without recovery group the difference was not significant ($p > 0.05$). When the level of ATP in sham-operated rat brains (Table 3.9) was compared with that measured in the two 20 minute control groups (Table 3.11) there were no significant differences ($F(1,22) = 0.26$, $p = 0.62$). The apparent trend for brain tissue ATP to increase during the recovery period in sham operated and control rats was not significant ($F(1,22) = 3.14$, $p = 0.09$).

When ATP was measured immediately after delivery there was a consistent and significant reduction in hypoxic brain tissue ATP in comparison with control rats (Tables 3.10 - 3.12 and figure 3.5). A 10 minute occlusion period (Table 3.10) caused a significant 55.5% reduction in brain tissue ATP ($p < 0.001$, unpaired t-test) and with an additional recovery period hypoxic rat brain tissue ATP remained significantly lower than control (43.0%, $p < 0.05$). Following a 20 minute occlusion period (Table 3.11) brain tissue ATP was significantly reduced by 69.6% ($p < 0.002$) and with a recovery period the mean 27.8% reduction in hypoxic brain tissue ATP was still significant ($p < 0.05$). The mean 78.0% reduction in brain tissue ATP after a 30 minute occlusion period (Table 3.12) was also significant ($p < 0.001$) and after a recovery period hypoxic rat brain tissue ATP was still significantly reduced by 70.5% ($p < 0.001$).

ANOVA of all the data collected at zero minutes after delivery supported the general trend for hypoxic rat brain tissue ATP to be lower than that in control rats ($F(1,74) = 107.73$, $p < 0.001$). The extent to which hypoxic brain tissue ATP was reduced appeared to be greater after a 30 minute occlusion period (see Figure 3.5) but ANOVA did not support this observation ($F(2,74) = 2.61$, $p = 0.08$). The recovery period had no visible effect on the level of control or hypoxic brain tissue ATP after a 30 minute occlusion which may reflect the influence of the long period of anaesthesia as well as the duration of the occlusion period.

The measurement of brain tissue ATP at one minute after delivery produced results similar to those observed at zero minutes after delivery (Tables 3.9 - 3.12) and the results are illustrated in figure 3.6. A 10 minute occlusion period caused a 51.9% reduction in ATP ($p < 0.02$, unpaired t-test, see Table 3.10) but after a recovery period hypoxic rat brain tissue ATP was not significantly different from control rats ($p > 0.05$). A 20 minute occlusion period led to a significant 67.4% reduction in hypoxic rat brain tissue ATP ($p < 0.02$, see Table 3.11) which was eliminated by the recovery period (27.8%, $p > 0.05$). At one minute after delivery the reduction in hypoxic rat brain tissue ATP associated with a 30 minute occlusion was not significant (55.8%, $p > 0.05$ - see table 3.12) but with a recovery period hypoxic rat ATP was significantly lower than in control rats (73.5%, $p < 0.001$). ANOVA of all brain tissue ATP levels measured at one minute after delivery supported the observation that ATP was significantly lower in hypoxic rat brains ($F(1,72) = 41.65$, $p < 0.001$). The increase in both control and hypoxic brain tissue ATP during the recovery period after a 20 minute occlusion was significant ($F(1,72) = 6.66$, $p = 0.01$) whereas recovery changes associated with the other occlusion periods were not significant at one minute after delivery.

The changes in brain tissue ATP during the first three hours of post-natal life are summarised in tables 3.9 - 3.12. ANOVA of data from the non-operated and sham-operated (with recovery) groups (Table 3.9) identified no significant change in ATP levels during early post-natal life ($F(3,40) = 1.64$, $p = 0.20$) but at all measured intervals the level of ATP in sham-operated rat brains was significantly higher than in non-

operated rat brains ($F(1,40) = 24.85$, $p < 0.001$) suggesting a long-lasting effect of the anaesthetic. There were also no significant temporal changes in post-natal ATP measured in control rat brains ($F(3,126) = 0.84$, $p = 0.47$). There was, however, a significant effect of time after delivery on the level of ATP in hypoxic rat brains ($F(3,126) = 12.43$, $p < 0.001$). Brain tissue ATP in hypoxic rats increase significantly between zero minutes and 60 minutes after delivery but there was no significant change between 60 and 180 minutes after delivery. The rise in hypoxic brain tissue ATP between zero and 60 minutes after delivery was particularly significant after a 30 minute occlusion period ($F(2,110) = 14.30$, $p < 0.001$) which might suggest that this occlusion period caused the greatest alteration in ATP metabolism.

TABLE 3.9 BRAIN TISSUE ATP (nmols/mg brain tissue) - SHAM OPERATED RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	MEAN BRAIN TISSUE ATP	NO. OF RATS
NON-OPERATED	0	1.71 ± 0.07	6
	1	1.96 ± 0.18	6
	60	1.98 ± 0.26	6
	180	2.17 ± 0.14	6
SHAM OPERATED (without recovery)	0	1.94 ± 0.24	6
	1	1.61 ± 0.13	6
	60		
	180		
SHAM OPERATED (with 20 mins recovery)	0	2.49 ± 0.13	6
	1	2.49 ± 0.12	6
	60	2.71 ± 0.34	6
	180	2.83 ± 0.15	6

The table summarises the data (mean ± s.e.m.) obtained from normal foetal rats delivered by Caesarian section (non-operated), from rats exposed to 25 minutes of anaesthesia (sham-operated-without recovery) and from rats exposed to anaesthesia followed by a recovery period of 20 minutes (sham-operated with recovery). Using the unpaired t-test no significant differences were found between the two sham-operated groups.

TABLE 3.10 BRAIN TISSUE ATP (nmols / mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 10 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue ATP	No. of Rats	Mean Brain Tissue ATP	No. of Rats	
10 MINS +	0	3.28 ± 0.31	6	1.46 ± 0.16	6	< 0.001
0 MINS	1	2.64 ± 0.29	6	1.27 ± 0.35	6	< 0.02
RECOVERY	60	2.71 ± 0.35	5	2.54 ± 0.26	5	n.s.
	180	2.70 ± 0.30	2	3.07 ± 0.30	3	n.a.
10 MINS +	0	1.93 ± 0.22	6	1.10 ± 0.23	6	< 0.05
20 MINS	1	1.93 ± 0.39	6	1.66 ± 0.22	6	n.s.
RECOVERY	60	1.95 ± 0.17	6	2.25 ± 0.23	6	n.s.
	180	2.17 ± 0.16	6	2.07 ± 0.19	6	n.s.

(n.s. = not significant, n.a. = not applicable)

The table contains a summary of all data (mean ± s.em.) obtained from those rats exposed to a 10 minute occlusion and their littermate controls. The bottom half of the table summarises data from rats exposed to a 20 minute recovery period after the occlusion.

TABLE 3.11 BRAIN TISSUE ATP (nmols / mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 20 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue ATP	No. of Rats	Mean Brain Tissue ATP	No. of Rats	
20 MINS +	0	2.14 ± 0.37	7	0.65 ± 0.08	7	< 0.002
0 MINS	1	1.90 ± 0.24	7	0.62 ± 0.12	7	< 0.01
RECOVERY	60					
	180					
20 MINS +	0	2.56 ± 0.24	7	1.75 ± 0.23	7	< 0.05
20 MINS	1	2.30 ± 0.14	6	1.66 ± 0.24	6	n.s.
RECOVERY	60	2.13 ± 0.07	5	1.46 ± 0.10	5	n.s.
	180	2.56 ± 0.23	4	2.42 ± 0.23	4	n.s.

(n.s. = not significant)

The table contains a summary of brain tissue ATP concentrations (mean ± s.e.m.) taken from rats exposed to a 20 minute occlusion (top half) and rats exposed to a 20 minute recovery period after the occlusion (bottom half) as well as their littermate controls.

TABLE 3.12 BRAIN TISSUE ATP (nmols/mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 30 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue ATP	No. of Rats	Mean Brain Tissue ATP	No. of Rats	
30 MINS +	0	2.68 ± 0.24	6	0.59 ± 0.08	6	< 0.001
0 MINS	1	2.24 ± 0.44	6	0.99 ± 0.37	6	n.s.
RECOVERY	60					
	180					
30 MINS +	0	2.44 ± 0.24	11	0.72 ± 0.20	11	< 0.001
20 MINS	1	2.00 ± 0.18	11	0.53 ± 0.26	11	< 0.001
RECOVERY	60	2.30 ± 0.19	6	2.45 ± 0.23	6	n.s.
	180					

(n.s. = not significant)

The table shows the mean ± s.e.m. brain tissue ATP found in rats which had been exposed to a 30 minute occlusion and in their littermate controls.

FIGURE 3.5 THE CHANGES IN BRAIN TISSUE ATP FOLLOWING AN IN UTERO HYPOXIC EPISODE

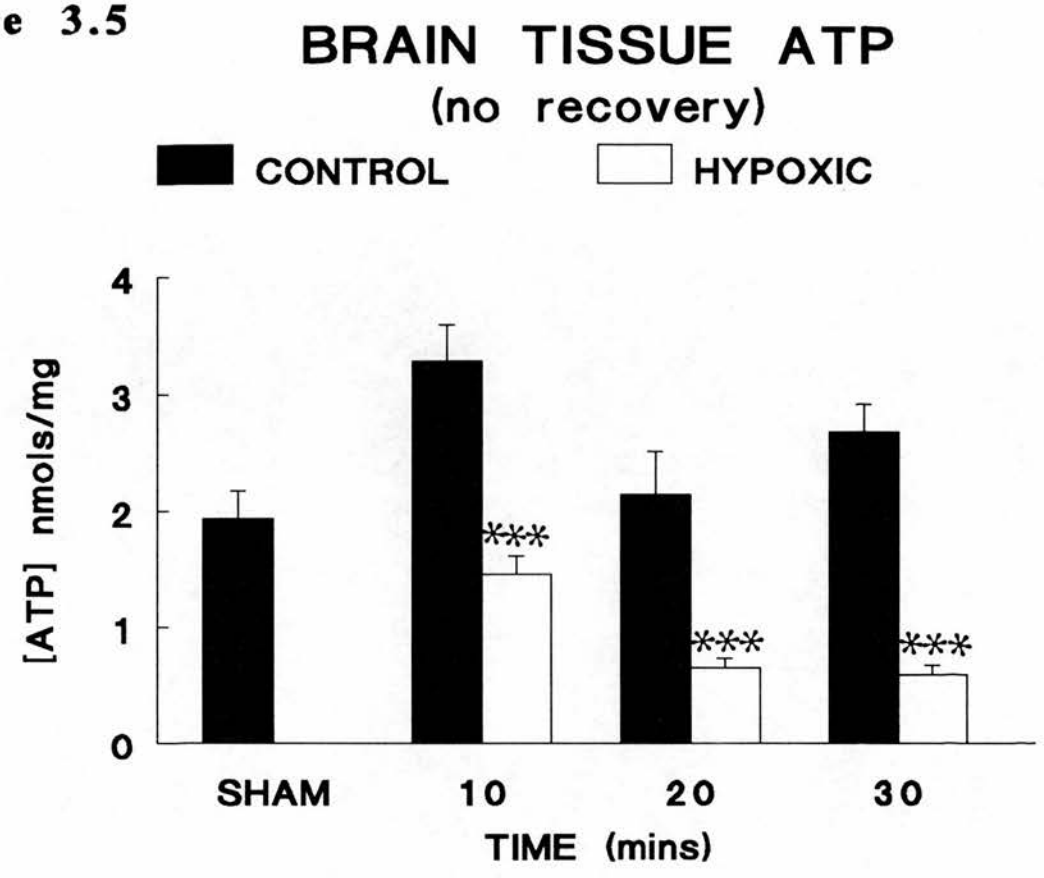
The histograms illustrate the concentration of ATP (nmols/mg brain tissue) in near-term foetal rat brains immediately after delivery by Caesarian section. Time refers to the duration of the occlusion period.

The sham operated rats were anaesthetised (25 minutes) and a midline incision performed but the uteroplacental vessels were not occluded. The bottom histogram represents data obtained from rats which were exposed to 20 minutes without anaesthesia (recovery) after completion of the occlusion period.

Following delivery of the rats the brains were removed and homogenised in $HClO_4$ at -10°C. The deproteinised tissue samples were assayed fluorimetrically according to the method of Lowry and Passoneau (1972) in order to detect the concentration of ATP.

The differences between control and hypoxic rat brain tissue ATP concentrations were statistically analysed using the unpaired t-test. * = $P < 0.05$, *** = $P < 0.005$.

Figure 3.5


BRAIN TISSUE ATP
(no recovery)
CONTROL
HYPOXIC
[ATP] nmols/mg
4
3
2
1
0
***
***
***
SHAM
10
20
30
TIME (mins)

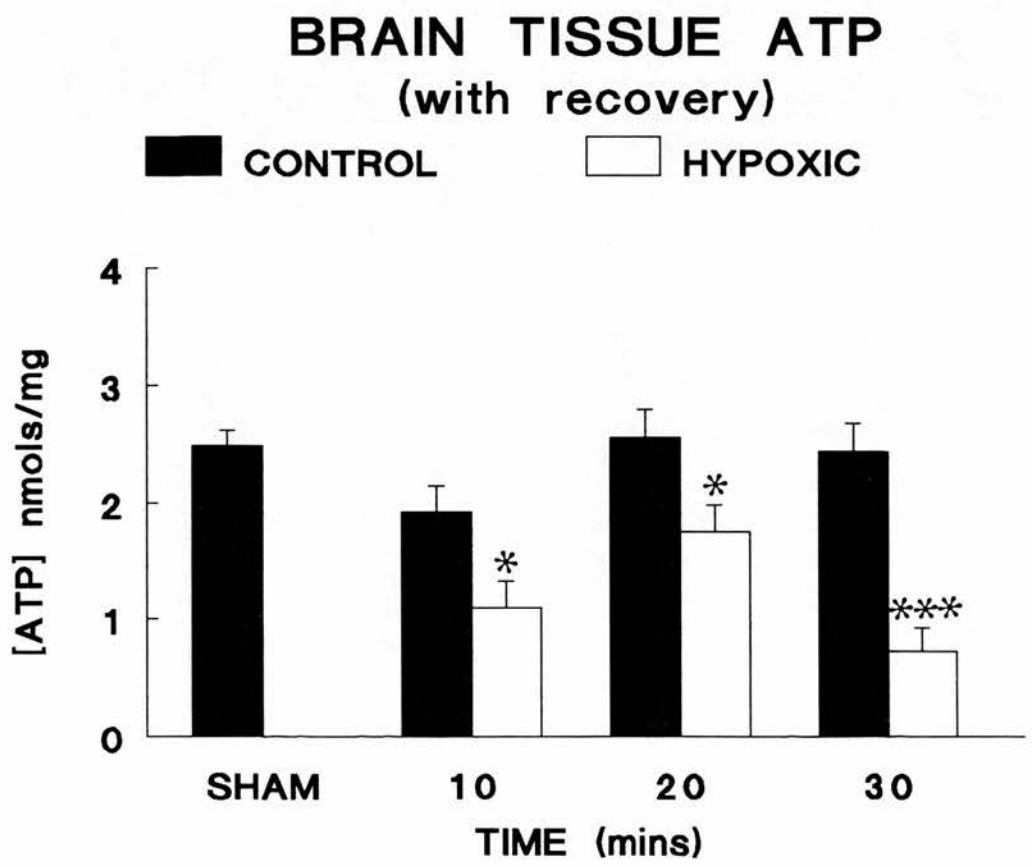
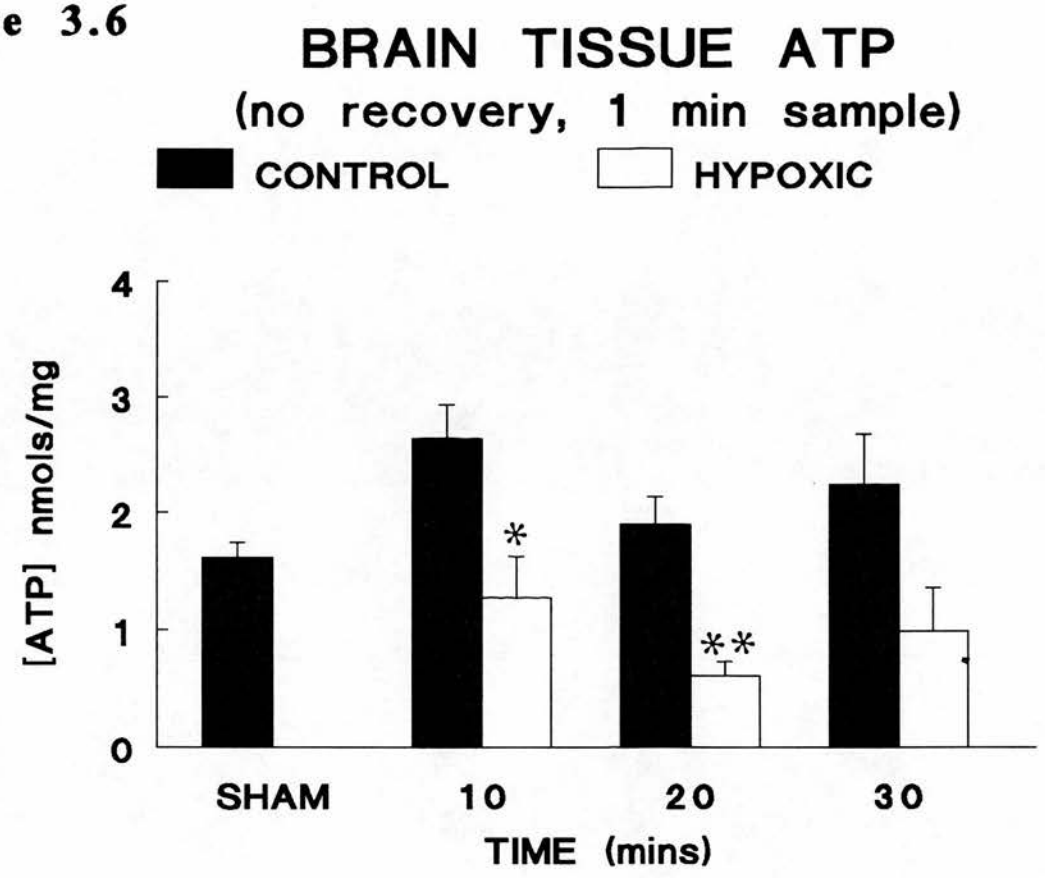

BRAIN TISSUE ATP
(with recovery)
CONTROL
HYPOXIC
[ATP] nmols/mg
4
3
2
1
0
*
*
***
SHAM
10
20
30
TIME (mins)

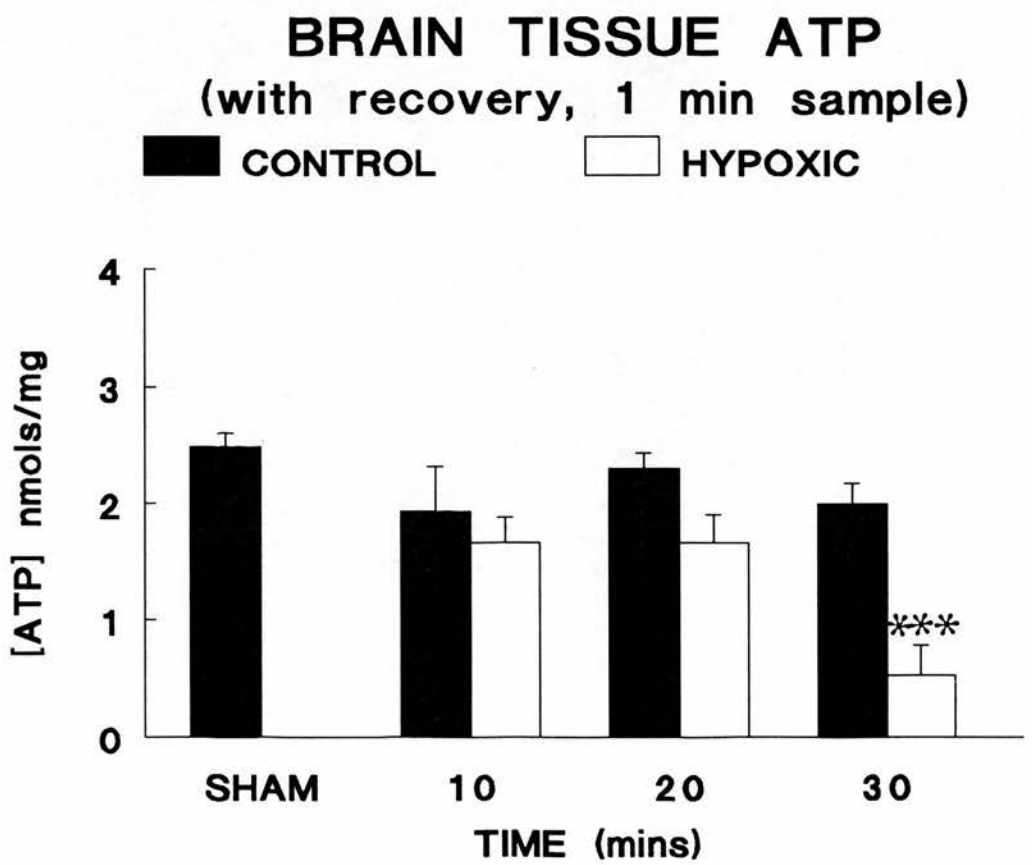
FIGURE 3.6 THE CHANGES IN BRAIN TISSUE ATP CONCENTRATIONS FOLLOWING AN IN UTERO HYPOXIC EPISODE MEASURED ONE MINUTE AFTER DELIVERY

The two histograms illustrate the concentration of ATP (nmols/mg brain tissue) measured in foetal rat brains one minute after delivery by Caesarian section. The time refers to the duration of the occlusion. The term 'with recovery' represents those animals exposed to a 20 minute period without anaesthesia following completion of the occlusion period.

For details of ATP measurement see Figure 3.5. All data were subjected to statistical analysis using the unpaired t-test. * $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$.

Figure 3.6


BRAIN TISSUE ATP
(no recovery, 1 min sample)
CONTROL
HYPOXIC
[ATP] nmols/mg
0
1
2
3
4
*
**
SHAM
10
20
30
TIME (mins)


BRAIN TISSUE ATP
(with recovery, 1 min sample)
CONTROL
HYPOXIC
[ATP] nmols/mg
0
1
2
3
4
***
SHAM
10
20
30
TIME (mins)

3.1.4 Brain Tissue Phosphocreatine (PCr)

A summary of all the data obtained from the measurement of brain tissue PCr in each rat can be found in tables 3.13 - 3.16. The changes in the concentration of PCr in hypoxic rat brains in comparison with control rat brains are illustrated in figures 3.7 and 3.8.

The ANOVA of non-operated and sham-operated rat brain tissue PCr measured at zero minutes after delivery (Table 3.13) found the level of PCr in the sham-operated - with recovery group to be significantly higher than in the non-operated group ($F(2,15) = 3.61$, $p = 0.05$). The level of PCr in the sham-operated - without recovery rat brains was not significantly higher than in the non-operated group and there was no significant difference between the two sham-operated groups. The difference between the non-operated and sham-operated-with recovery group suggests an increase in brain PCr in response to the anaesthetic but with no difference between the two sham-operated groups a recovery period, without anaesthetic, had no significant effect either to further increase or reduce accumulation of brain tissue PCr. The comparison of the level of brain tissue PCr in the sham-operated groups (Table 3.13) with that in the two 20-minute control groups (Table 3.15) found no significant difference ($F(1,22) = 0.55$, $p = 0.47$). The trend for brain tissue PCr to be higher in both groups of rats exposed to a recovery period was not significant ($F(1,22) = 3.25$, $p = 0.86$).

The measurement of control and hypoxic rat brain tissue PCr at zero minutes after delivery showed a consistent reduction in PCr following an *in utero* occlusion (Figure 3.7). An occlusion period of 10 minutes (Table 3.14) resulted in a 56.0% reduction in PCr ($p > 0.05$, unpaired t-test) and with an additional recovery period hypoxic PCr was reduced by 69.7% in comparison with control PCr ($p < 0.05$). A 20 minute occlusion (Table 3.15) caused a mean 82.9% reduction in brain tissue PCr ($p < 0.02$) and after the recovery period the hypoxic rat brain PCr was still significantly lower than in control rats (72.2%, $p < 0.01$). A 30 minute occlusion period was

associated with a 84.0% mean reduction in PCr ($p < 0.01$, see Table 3.16) and when followed by a 20 minute recovery period hypoxic brain tissue PCr was still significantly reduced (mean = 77.5%, $p < 0.001$).

ANOVA of control and hypoxic brain tissue PCr measured at zero minutes after delivery supported the consistent and significant decrease in hypoxic brain PCr in comparison with control PCr ($F(1,74) = 67.78$, $p < 0.001$). Although PCr appeared to fall during recovery after a 10 minute surgical procedure and rise after a 20 minute surgical procedure (Figure 3.7) the recovery period had no significant effect on control or hypoxic rat brain PCr ($F(1,74) = 0.11$, $p = 0.75$). The lack of significant influence of the recovery period on brain PCr levels may be a feature of the wide variance in measured concentrations of PCr observed, particularly in control rats.

The extent to which PCr declined in hypoxic rat brains was not significantly related to the duration of the occlusion period ($F(2,74) = 0.26$, $p = 0.77$) which means that the majority of the change in PCr occurred during the early part of the *in utero* occlusion.

At one minute after delivery hypoxic brain tissue PCr concentrations tended to be lower than those observed in control rat brains (Figure 3.8). The reduction in hypoxic brain tissue PCr after a 10 minute occlusion period (Table 3.14) was not significant either without (48.7%, $p > 0.05$) or with a recovery period (53.9%, $p > 0.05$). The reduction in hypoxic brain tissue PCr after a 20 minute occlusion period (Table 3.15) was significant without a recovery period (81.3%, $p < 0.05$) but with a recovery period differences between control and hypoxic rats were minimal (35.7% increase, $p > 0.05$). A 30 minute occlusion without a recovery period (Table 3.16) caused a 29.03% reduction in PCr ($p > 0.05$) and with a recovery period hypoxic brain PCr was significantly reduced by 75.0% ($p < 0.002$). The performance of ANOVA on all control and hypoxic rat brain PCr measured at one minute after delivery also exposed significant reductions in hypoxic brain tissue PCr in comparison with control rats ($F(1,72) = 23.46$, $p < 0.001$).

As with PCr levels measured at zero minutes after delivery, at one minute after delivery there was no overall, significant effect of the recovery period ($F(1,72) = 0.25$, $p = 0.62$). The increase in both control and hypoxic rat brain PCr during the recovery period after a 20 minute surgical procedure, however, was significant ($F(1,72) = 8.56$, $p = 0.005$) which supports the finding that differences between control and hypoxic rats were minimal when the recovery period followed a 20 minute occlusion.

Although the reductions in PCr associated with 30 minute occlusions were not significantly different to those associated with 10 or 20 minute occlusions, at either zero or one minute after delivery, the fact that PCr did not increase during the recovery period suggests a prolonged influence of the 30 minute occlusion after the clips had been removed.

When brain tissue PCr was measured at 0, 1, 60 and 180 minutes after delivery an increase between 1 and 60 minutes was evident (see Tables 3.13 - 3.16). The performance of ANOVA on brain PCr measured in the early post-natal period in non-operated and sham-operated rats (with recovery - Table 3.13) exposed a significant effect of time after delivery ($F(3,40) = 16.26$, $p < 0.001$). Post-hoc comparisons showed that in non-operated rat brains there was a significant rise in PCr between 0 and 60 minutes after delivery but differences between 0 and 1 and between 60 and 180 minutes after delivery were not significant.

For sham-operated rats post-hoc comparisons showed that differences between zero and 60 or zero and 180 minutes after delivery were not significant, however, the rise in PCr between one and 60 minutes was significant. The level of brain tissue PCr in sham-operated rats fell between zero (0.73 ± 0.12 nmols/mg brain tissue) and one (0.50 ± 0.08 nmols/mg brain tissue) minute after delivery and, although this fall was not significant, this accounts for the significant rise in PCr between one and 60 minutes after delivery (Table 3.13). Since PCr did not fall during the first minute of the post-natal life in non-operated rats it may be inferred that rats exposed to anaesthetic are vulnerable to a transient reduction in PCr, possibly caused by a delay in the onset of spontaneous gasping.

The ANOVA of postnatal PCr in control and hypoxic brains (Tables 3.14 - 3.16) highlighted a similar trend for PCr to decrease between zero and one minutes after delivery in control rats ($F(3,126) = 6.78$, $p < 0.001$). PCr may decline during the first minute of post-natal life because of a transient hypoxic insult before the onset of spontaneous respiration but in occluded rats, which had already been exposed to a hypoxic insult, there was no further decrease in PCr between zero and one minutes after delivery. The decline in control but not hypoxic brain tissue PCr during the first minute may account for the less significant differences between control and hypoxic rats observed at one minute after delivery (see above). A significant influence of time after delivery on all rats ($F(3,126) = 52.52$, $p < 0.001$) reflected the rise in brain tissue PCr in the first hour but no further change occurred between 60 and 180 minutes after delivery.

3.1.5 Brain Tissue ADP

The previous results had shown that the 30 minute occlusion period produced the most significant changes in brain tissue metabolites. ADP was, therefore, only measured in those samples taken after a 30 minute occlusion. Table 3.17 summarises the data obtained from the 30 minute samples which were assayed and figure 3.9 illustrates the differences between the control and the hypoxic brain tissue samples.

As table 3.17 shows, a 30 minute occlusion period was associated with a significantly higher level of brain tissue ADP in hypoxic rat brains at zero minutes after delivery (115.6%, $p < 0.01$, unpaired t-test) but with an additional recovery period there was no significant difference between control and hypoxic rats (61.1%, $p > 0.05$). ANOVA of control and hypoxic ADP levels also highlighted a significantly higher level of ADP in hypoxic rats ($F(1,12) = 20.30$, $p = 0.001$).

At one minute after delivery ADP was also elevated in hypoxic rat brains but this was only significant after a recovery period (44.8%, $p < 0.05$). The recovery period did not, however, have a significant effect on either control or hypoxic rat brain tissue ADP

($F(1,12) = 3.30$, $p = 0.11$) which suggests that the 30 minute surgical procedure had an influence on ADP levels which persisted when both the occlusion and the anaesthetic were removed.

3.1.6 Brain Tissue AMP

As with brain tissue ADP it was decided to measure brain tissue AMP only in those rats which had been exposed to a 30 minute occlusion period. Table 3.18 summarises the levels of brain tissue AMP found in control and hypoxic rats and the data are illustrated in figure 3.10. Brain tissue AMP was significantly elevated following a 30 minute occlusion without (564.0%, $p < 0.001$ unpaired t-test) and with a recovery period (579.0%, $p < 0.05$). The accumulation of AMP was also evident at one minute after delivery (see Table 3.18). ANOVA did not expose a significant effect of the recovery period on control or hypoxic rat brain AMP ($F(1,12) = 3.36$, $p = 0.92$) which again suggests that the influence of the 30 minute procedure persists for some time.

TABLE 3.13 BRAIN TISSUE PHOSPHOCREATINE (PCr) (nmols/mg brain tissue) - SHAM OPERATED RATS

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	MEAN BRAIN TISSUE PCr	NO. OF RATS
NON-OPERATED	0	0.37 ± 0.08	6
	1	0.58 ± 0.15	6
	60	1.25 ± 0.22	6
	180	1.31 ± 0.19	6
SHAM OPERATED (without recovery)	0	0.55 ± 0.08	6
	1	0.29 ± 0.05	6
	60		
	180		
SHAM OPERATED (with 20 mins recovery)	0	0.73 ± 0.12	6
	1	0.50 ± 0.08	6
	60	1.30 ± 0.19	6
	180	1.27 ± 0.10	6

The table shows a summary of brain tissue PCr (mean ± s.e.m.) found in normal foetal rats delivered by Caesarian section (non-operated) in rats exposed to 25 minutes of anaesthesia (sham-operated-without recovery) and in rats exposed to a 20 minute recovery period after anaesthesia (sham operated-with recovery).
Using the unpaired t-test no significant differences were found between the two sham-operated groups.

TABLE 3.14 BRAIN TISSUE PHOSPHOCREATINE (PCr) (nmols/mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 10 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue PCr	No. of Rats	Mean Brain Tissue PCr	No. of Rats	
10 MINS +	0	0.91 ± 0.21	6	0.40 ± 0.08	6	n.s.
0 MINS	1	0.78 ± 0.13	6	0.40 ± 0.11	6	n.s.
RECOVERY	60	1.41 ± 0.14	5	1.33 ± 0.15	5	n.s.
	180	1.59 ± 0.15	2	1.83 ± 0.15	2	n.a.
10 MINS +	0	0.76 ± 0.19	6	0.23 ± 0.06	6	< 0.05
20 MINS	1	0.52 ± 0.11	6	0.24 ± 0.08	6	n.s.
RECOVERY	60	0.96 ± 0.16	6	1.23 ± 0.13	6	n.s.
	180	1.15 ± 0.16	6	1.12 ± 0.11	6	n.s.

(n.s. = not significant, n.a. = not applicable)

The table summarises data (mean ± s.e.m.) obtained from rats exposed to a 10 minute occlusion without a recovery period (top half), those exposed to 10 minutes occlusion with a recovery period (bottom half) and their littermate controls.

TABLE 3.15 BRAIN TISSUE PHOSPHOCREATINE (PCr) (nmols/mg brain tissue). A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 20 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue PCr	No. of Rats	Mean Brain Tissue PCr	No. of Rats	
20 MINS +	0	0.70 ± 0.18	7	0.12 ± 0.06	7	< 0.02
0 MINS	1	0.48 ± 0.12	7	0.09 ± 0.03	7	< 0.05
RECOVERY	60					
	180					
20 MINS +	0	0.97 ± 0.17	7	0.27 ± 0.07	7	< 0.01
20 MINS	1	0.70 ± 0.20	6	0.45 ± 0.09	6	n.s.
RECOVERY	60	1.34 ± 0.15	5	1.15 ± 0.12	5	n.s.
	180	1.47 ± 0.17	4	1.48 ± 0.21	4	n.s.

(n.s. = not significant)

The table summarises brain tissue PCr (mean ± s.e.m.) found in rats following a 20 minute occlusion and in their littermate controls. The bottom portion of the table shows data from those rats exposed to a 20 minute recovery period after the occlusion.

TABLE 3.16 BRAIN TISSUE PHOSPHOCREATINE (PCr) (nmols/mg brain tissue). A COMPARISON BETWEEN CONTROL RATS AND RATS EXPOSED TO A 30 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue PCr	No. of Rats	Mean Brain Tissue PCr	No. of Rats	
30 MINS +	0	0.75 ± 0.17	6	0.12 ± 0.06	6	< 0.01
0 MINS	1	0.31 ± 0.10	6	0.22 ± 0.13	6	n.s.
RECOVERY	60					
	180					
30 MINS +	0	0.80 ± 0.10	11	0.18 ± 0.05	11	< 0.001
20 MINS	1	0.40 ± 0.07	11	0.10 ± 0.05	11	< 0.002
RECOVERY	60	1.09 ± 0.08	6	1.42 ± 0.16	6	n.s.
	180					

(n.s. = not significant)

The table shows a summary of all brain tissue PCr levels found in rats following a 30 minute occlusion (top half) and in rats which were exposed to a 20 minute recovery period after the occlusion (lower half).

FIGURE 3.7 THE CHANGES IN BRAIN TISSUE PCr FOLLOWING AN IN UTERO HYPOXIC EPISODE

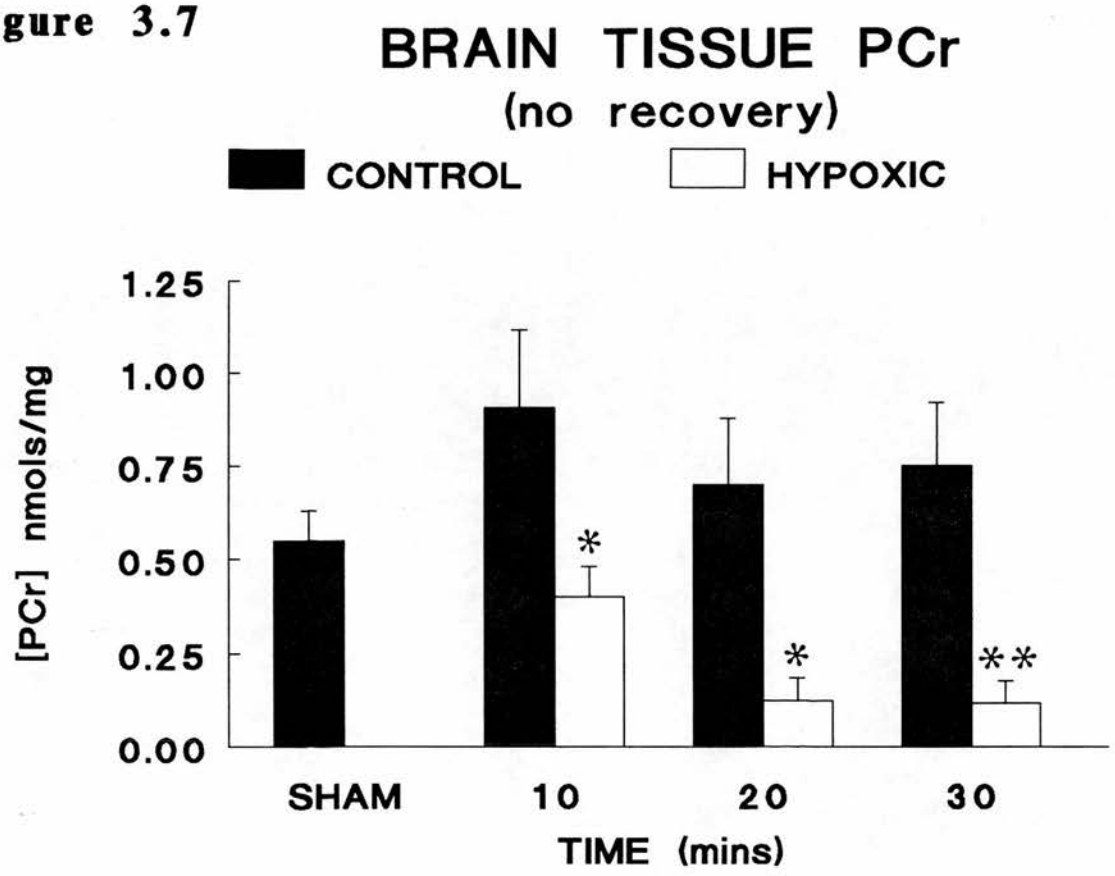
The histograms illustrate the concentration of PCr (nmols/mg brain tissue) found in foetal rat brains immediately after delivery by Caesarian section. Time refers to the duration of the occlusion period.

During a 25 minute period of anaesthesia for the sham procedure a midline incision was performed on the mothers and the uterine horns exposed but no uteroplacental vessels were occluded. The bottom histogram represents the data obtained from rats exposed to a 20 minute recovery period after the occlusion.

Following delivery of the foetal rat the brain was removed and homogenised in $HClO_4$ at $-10^{o}C$. The deproteinised tissue sample was assayed fluorimetrically according to the method of Lowry and Passoneau (1972) to obtain the concentration of PCr present in the rat brain.

All control and hypoxic levels of brain tissue PCr were analysed statistically using the unpaired t-test. * = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.005$.

Figure 3.7


BRAIN TISSUE PCr
(no recovery)
CONTROL
HYPOXIC
[PCr] nmols/mg
1.25
1.00
0.75
0.50
0.25
0.00
*
*
**
SHAM
10
20
30
TIME (mins)

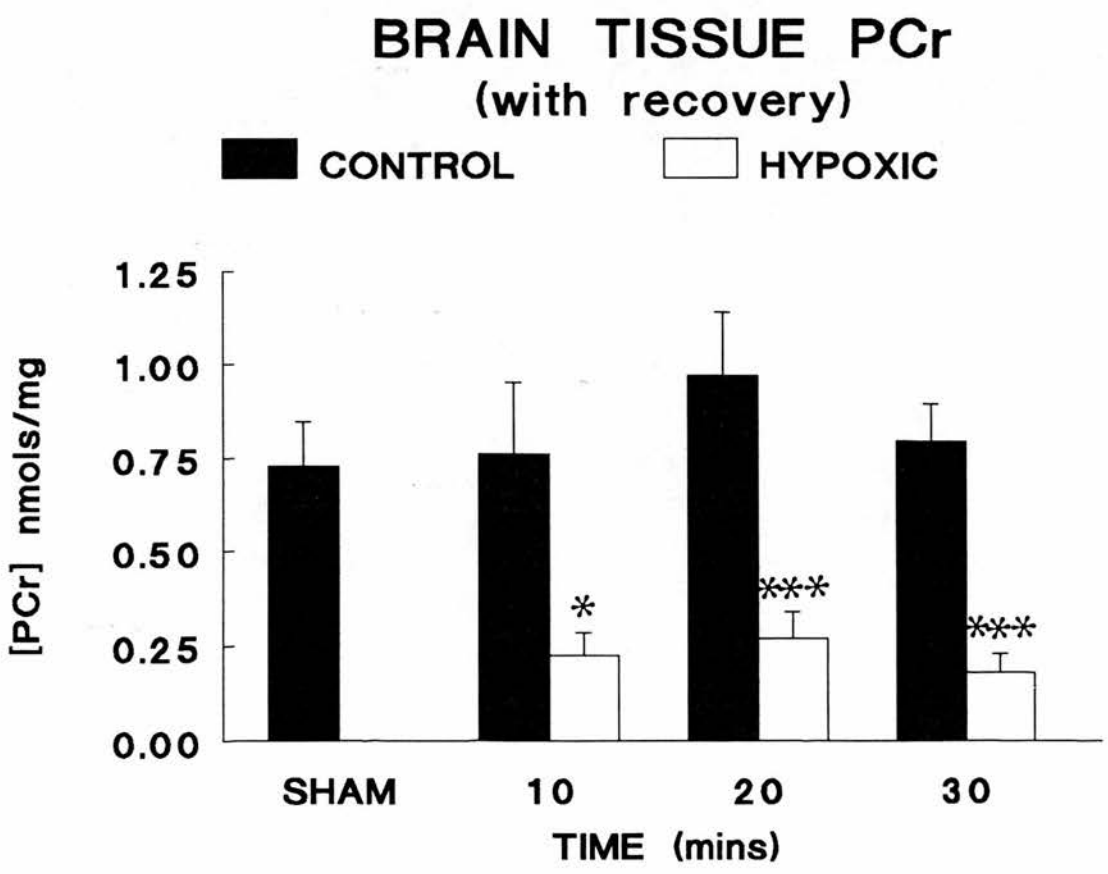

BRAIN TISSUE PCr
(with recovery)
CONTROL
HYPOXIC
[PCr] nmols/mg
1.25
1.00
0.75
0.50
0.25
0.00
*
***
***
SHAM
10
20
30
TIME (mins)

FIGURE 3.8 THE CHANGES IN BRAIN TISSUE PCr FOLLOWING AN IN UTERO HYPOXIC EPISODE MEASURED ONE MINUTE AFTER DELIVERY

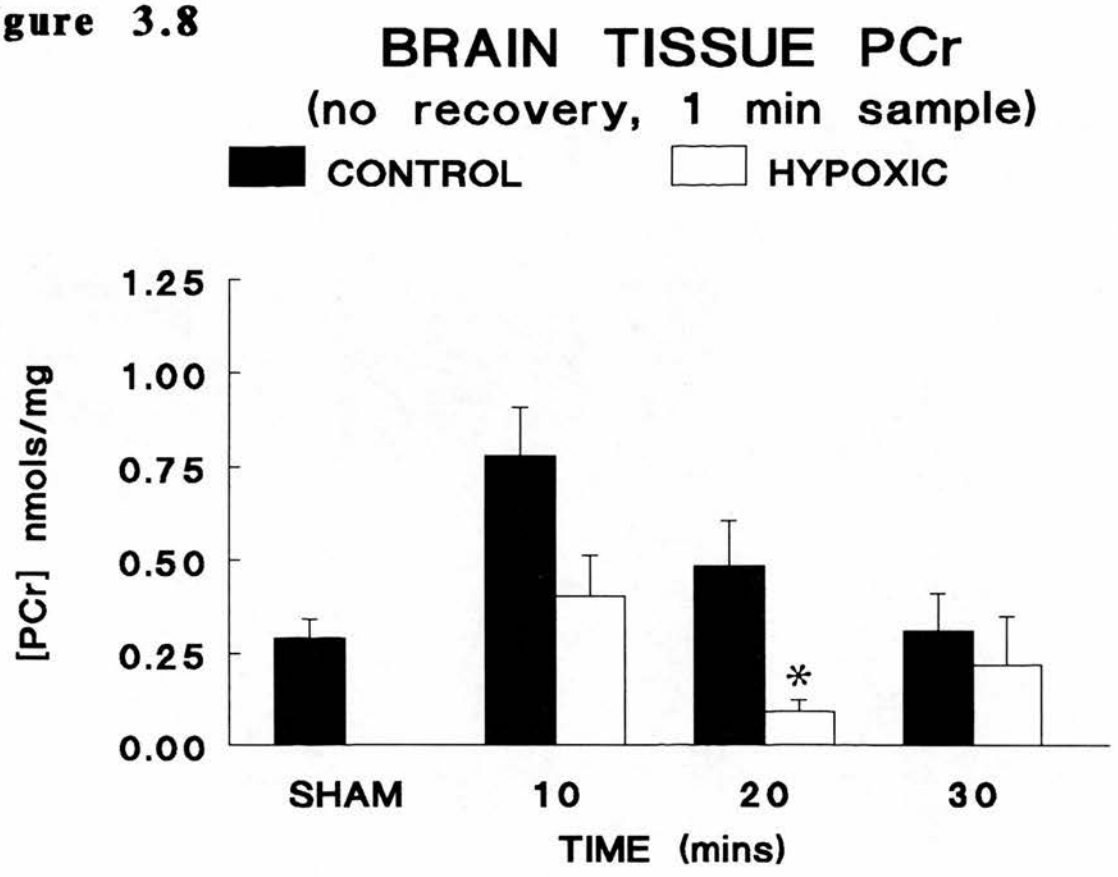
The two histograms illustrate the concentrations of PCr (nmols/mg brain tissue) in foetal rat brains which were taken one minute after delivery by Caesarian section. Time refers to the duration of the occlusion period.

The bottom histogram illustrates the levels of brain tissue PCr in rats which were exposed to a 20 minute recovery period following the occlusion.

For a description of procedures for measurement of PCr see figure 3.7.

All data were compared using the unpaired t-test. * = $P < 0.05$, *** = $P < 0.005$.

Figure 3.8


BRAIN TISSUE PCr
(no recovery, 1 min sample)
CONTROL
HYPOXIC
[PCr] nmols/mg
1.25
1.00
0.75
0.50
0.25
0.00
*
SHAM
10
20
30
TIME (mins)

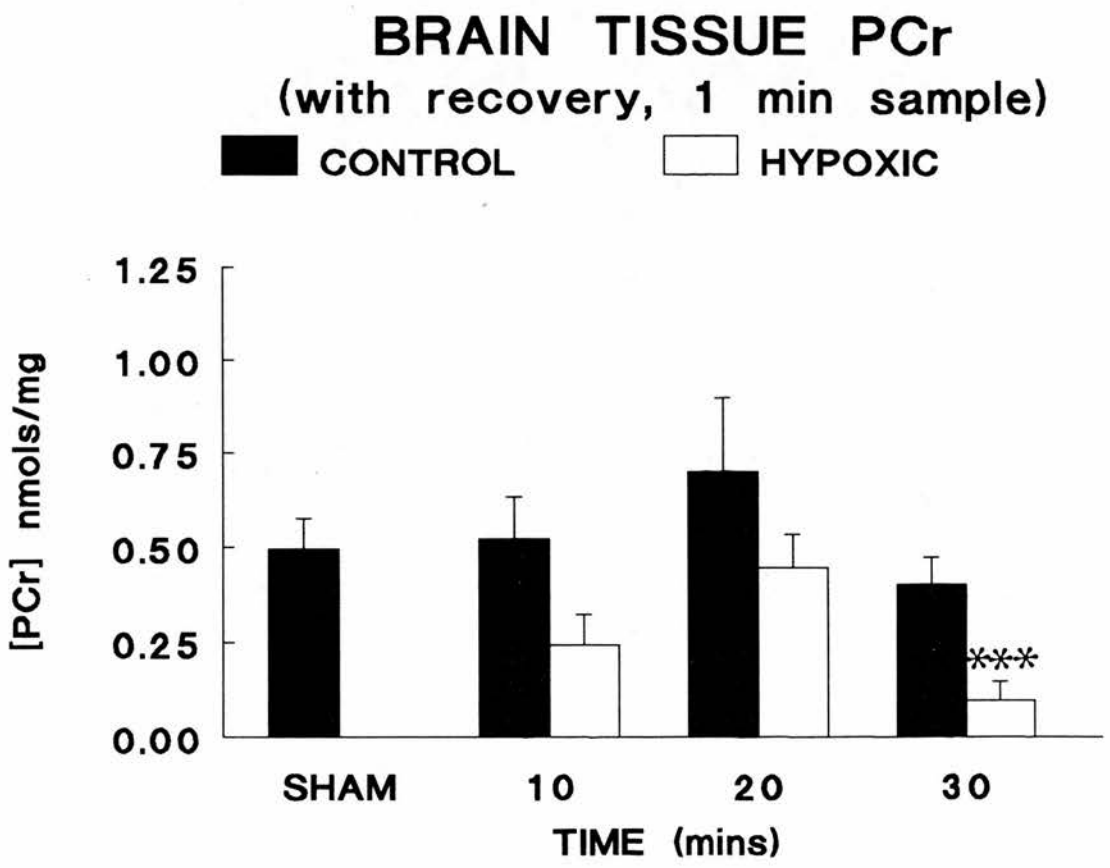

BRAIN TISSUE PCr
(with recovery, 1 min sample)
CONTROL
HYPOXIC
[PCr] nmols/mg
1.25
1.00
0.75
0.50
0.25
0.00
***
SHAM
10
20
30
TIME (mins)

TABLE 3.17 BRAIN TISSUE ADP (nmols/mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND HYPOXIC RATS EXPOSED TO A 30 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue ADP	No. of Rats	Mean Brain Tissue ADP	No. of Rats	
30 MINS +	0	0.77 ± 0.02	4	1.66 ± 0.11	4	< 0.01
0 MINS	1	0.87 ± 0.07	4	1.26 ± 0.13	4	n.s.
RECOVERY	60					
	180					
30 MINS +	0	0.72 ± 0.06	4	1.17 ± 0.27	4	n.s.
20 MINS	1	0.72 ± 0.03	4	1.16 ± 0.13	4	< 0.05
RECOVERY	60					
	180					

(n.s. = not significant)

The table shows the mean ± s.e.m. concentrations of brain tissue ADP in foetal rats following a 30 minute occlusion period. The lower portion shows data from rats which were exposed to a 20 minute recovery period after the occlusion was complete.

FIGURE 3.9 BRAIN TISSUE ADP CHANGES FOLLOWING IN UTERO HYPOXIA

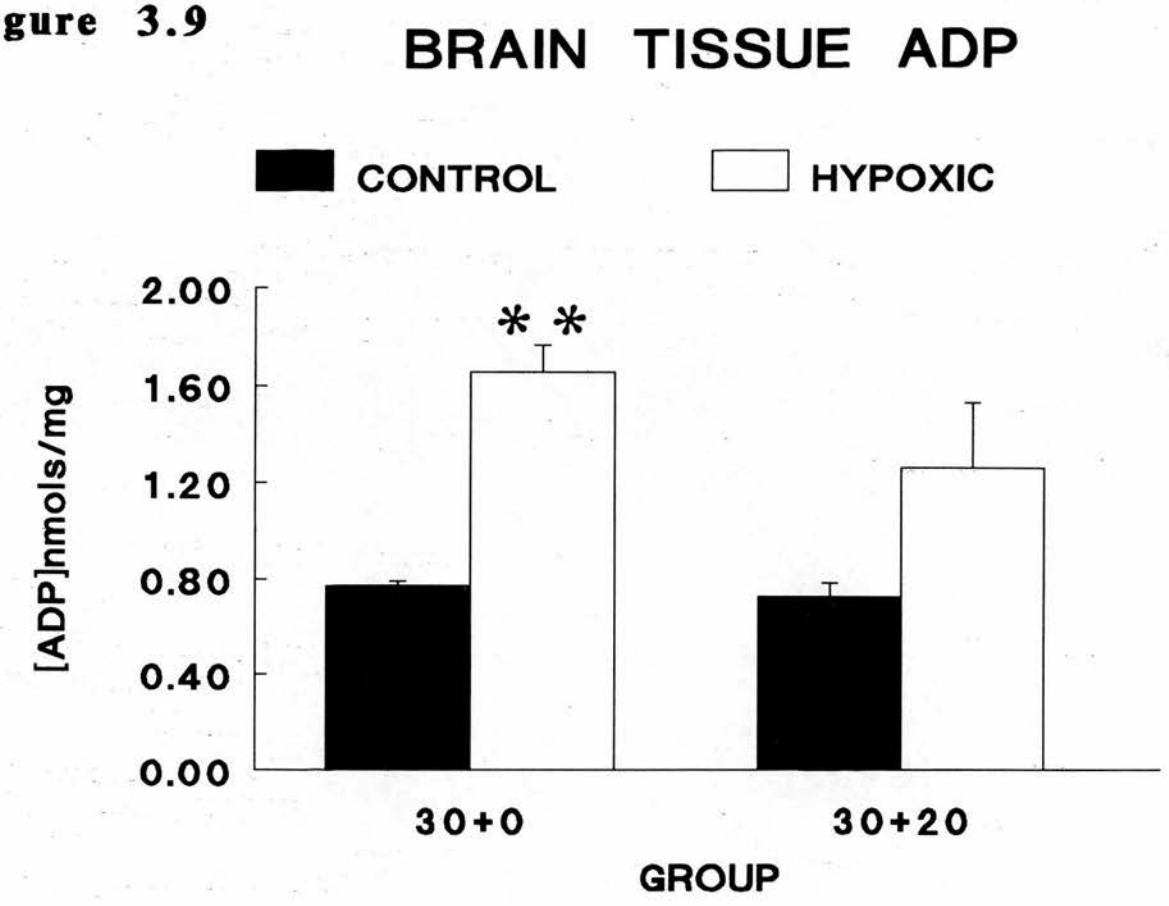
The histograms represent the levels of brain tissue ADP in control and hypoxic rat brains following a uteroplacental vessel occlusion of 30 minutes. The histograms on the right illustrate ADP levels when the occlusion was followed by a 20 minute recovery period.

The top histograms show ADP levels immediately after delivery and the bottom histograms represent ADP levels found one minute after delivery by Caesarian section.

Only 30 minute samples were assayed because this occlusion period had been associated with the greatest changes in other measured metabolites. The deproteinised tissue samples were assayed fluorimetrically according to the method of Lowry and Passoneau (1972) and the level of ADP was proportional to the decrease in fluorescence.

The control and hypoxic brain tissue ADP concentrations were statistically compared using the unpaired t-test: ($* = P < 0.05$, $** = P < 0.01$).

Figure 3.9


BRAIN TISSUE ADP
CONTROL
HYPOXIC
2.00
1.60
1.20
0.80
0.40
0.00
[ADP]nmols/mg
* *
30+0
30+20
GROUP

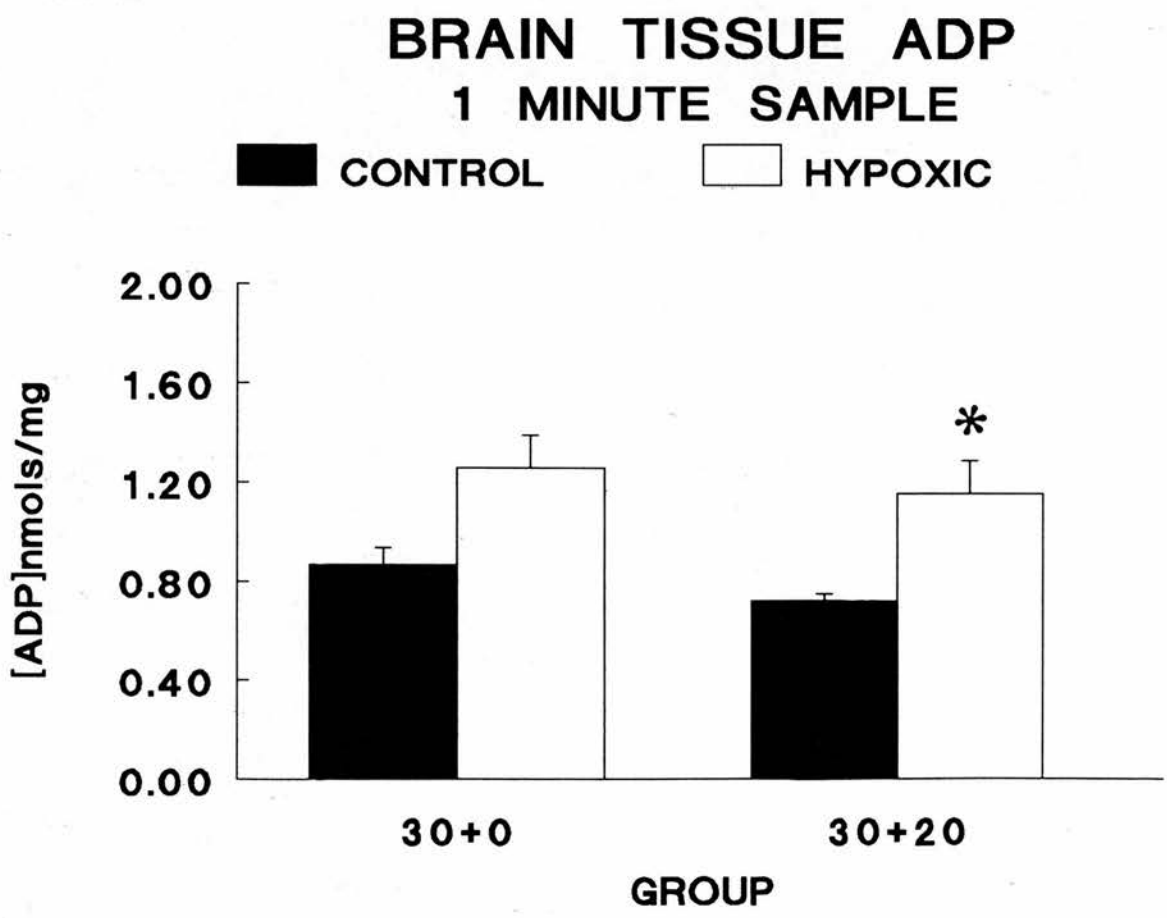
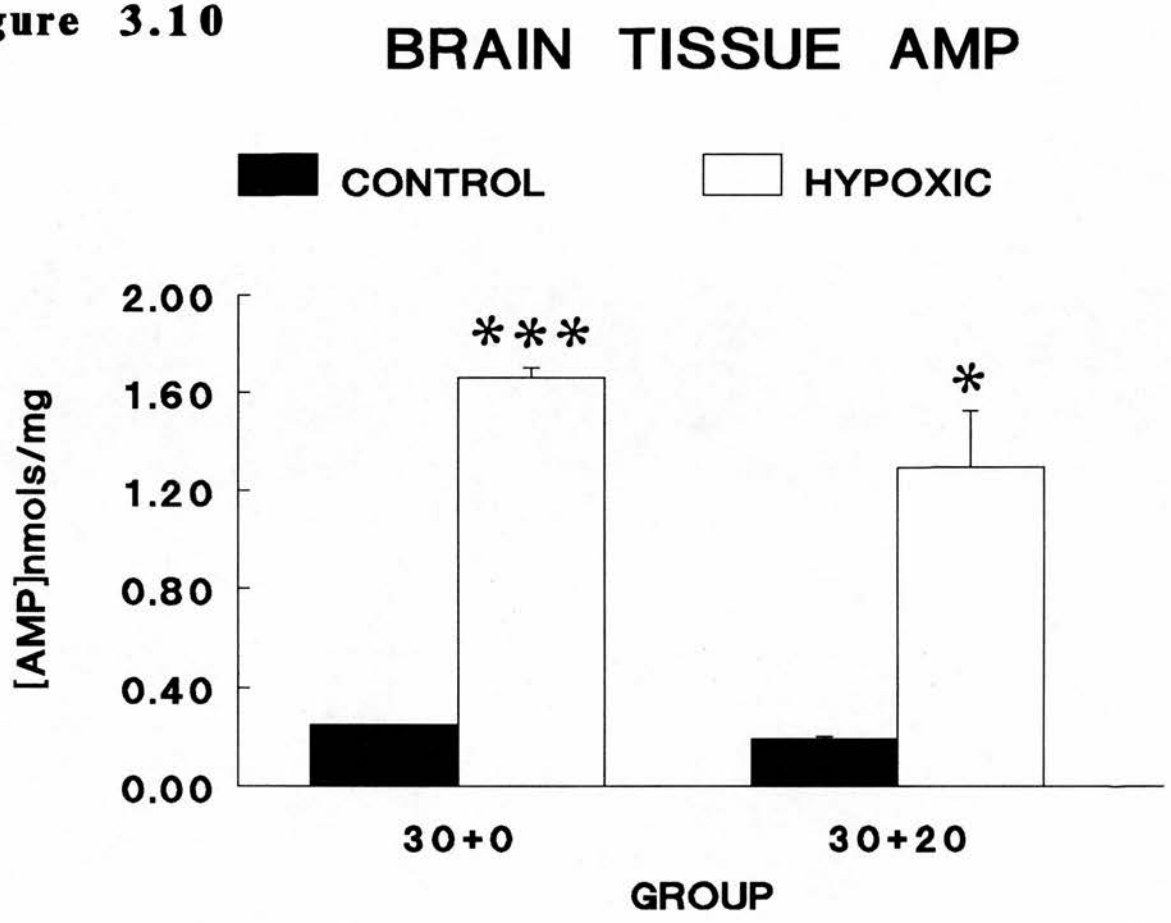

BRAIN TISSUE ADP
1 MINUTE SAMPLE
CONTROL
HYPOXIC
2.00
1.60
1.20
0.80
0.40
0.00
[ADP]nmols/mg
*
30+0
30+20
GROUP

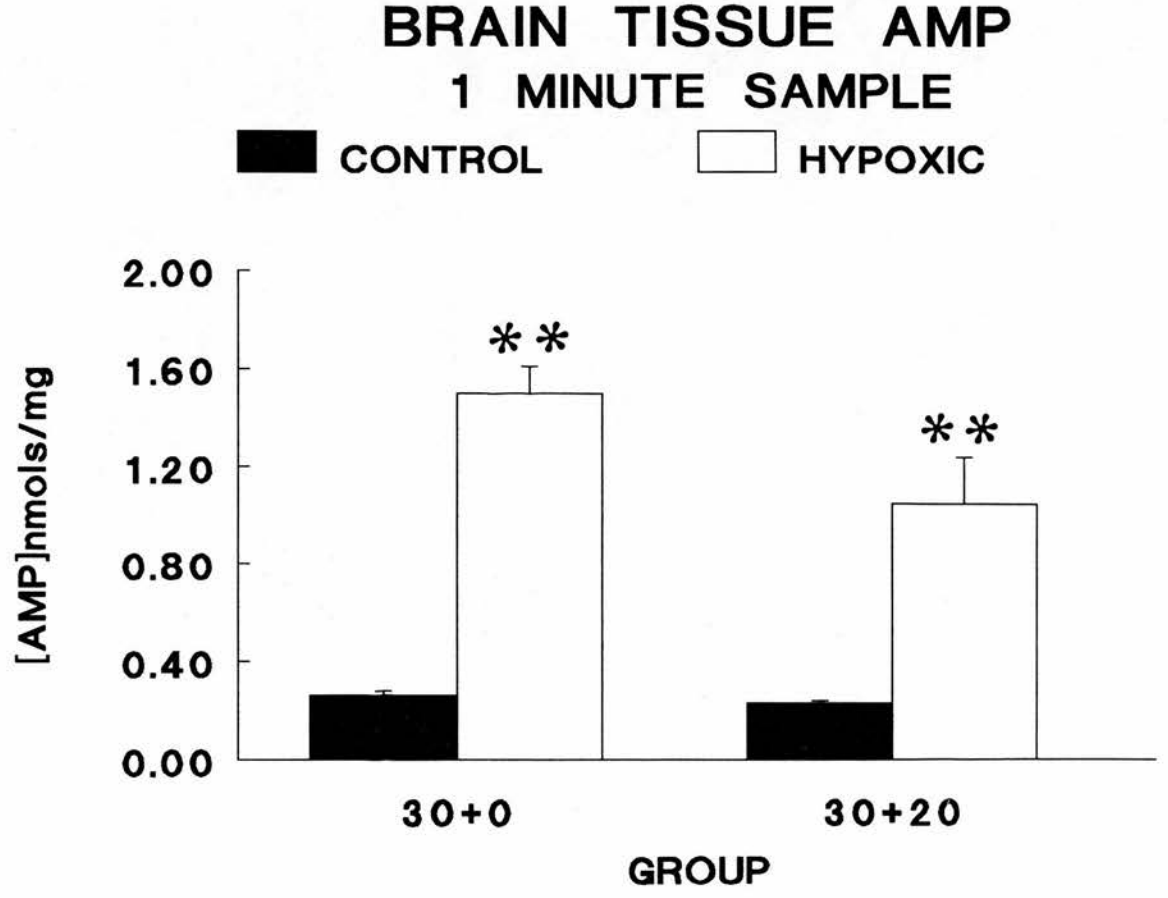
TABLE 3.18 BRAIN TISSUE AMP (nmols/mg brain tissue) A COMPARISON BETWEEN CONTROL RATS AND HYPOXIC RATS EXPOSED TO A 30 MINUTE OCCLUSION

EXPERIMENTAL GROUP	TIME AFTER DELIVERY (mins)	CONTROL RATS		HYPOXIC RATS		UNPAIRED T-TEST p
		Mean Brain Tissue AMP	No. of Rats	Mean Brain Tissue AMP	No. of Rats	
30 MINS +	0	0.25 ± 0.00	4	1.66 ± 0.04	4	< 0.001
0 MINS	1	0.26 ± 0.02	4	1.50 ± 0.11	4	< 0.002
RECOVERY	60					
	180					
30 MINS +	0	0.19 ± 0.01	4	1.29 ± 0.23	4	< 0.05
20 MINS	1	0.23 ± 0.01	4	1.05 ± 0.19	4	< 0.01
RECOVERY	60					
	180					

The table summarises data (mean ± s.e.m.) obtained from rats which were exposed to a 30 minute occlusion and their littermate controls. Data from rats which were exposed to a recovery period after the occlusion are shown in the lower portion.

Figure 3.10


BRAIN TISSUE AMP
CONTROL
HYPOXIC
[AMP]nmols/mg
2.00
1.60
1.20
0.80
0.40
0.00
***
*
30+0
30+20
GROUP


BRAIN TISSUE AMP
1 MINUTE SAMPLE
CONTROL
HYPOXIC
[AMP]nmols/mg
2.00
1.60
1.20
0.80
0.40
0.00
**
**
30+0
30+20
GROUP

3.1.7 Discussion

The alterations in foetal rat brain tissue metabolites following occlusion of the uteroplacental vessels can be taken to indicate a severe metabolic disruption in the brain during the occlusion (Siesjö, 1984; Duffy *et al*, 1975). Restriction of the maternal arterial supply to the foetal rats in the occluded horn would appear to have reduced the oxygen supply to the foetal rat brains thereby stimulating anaerobic glycolysis and causing an accumulation of brain tissue lactate. The reduction in brain tissue glucose was also consistent with a stimulation of glycolytic flux. The fact that brain tissue lactate accumulated further following longer occlusion periods may indicate a continued glycolytic flux and therefore a continued supply of glucose to the brain. Brain tissue glucose was reduced in all hypoxic rats but as the proportion by which glucose fell between a 10 and 30 minute occlusion period did not change then there might have been a continued supply of glucose to the foetal brain. Previous experiments have shown that in the perinatal animal regional cerebral glucose utilisation rises during the hypoxia and release of glucose from cardiac and cerebral glycogen stores in the foetal rat is believed to sustain anaerobic glycolysis (Vannucci *et al*, 1989; Bomont *et al*, 1992; Burchfield *et al*, 1990; Vannucci and Duffy, 1976; Dawes *et al*, 1959).

The persistent lactate accumulation is also an indication of reduced oxidative metabolism but since data for brain tissue pyruvate was not available and the lactate/pyruvate ratio cannot be calculated this conclusion must be speculative. However, it is known that lactate is often the preferred substrate for oxidative metabolism in the foetal rat brain and so the continued rise in lactate with the longer occlusion periods might indicate reduced oxidative capabilities and therefore a hypoxic insult (Hawkins *et al*, 1971; Cremer, 1982).

The rapid decline in brain tissue PCr is also indicative of a hypoxic insult and the fact that PCr did not decline further with the longer occlusion periods is consistent with previous experiments (Vannucci and Duffy, 1976). The metabolic changes associated with hypoxia are thought to stimulate the production of ATP via sources alternative to

anaerobic glycolysis which can only yield 2 moles of ATP for every mole of glucose. Creatine kinase catalyses the conversion of PCr and ADP to ATP and stimulation of this enzyme may account for the rapid and early decline in PCr (Siesjö, 1978; Duffy *et al*, 1975).

It is widely accepted that ATP levels are not greatly affected when brief periods of hypoxia occur in isolation but in this model of uteroplacental vessel occlusion brain tissue ATP was reduced considerably during the occlusion (Siesjö, 1984; Vannucci and Duffy, 1976). The fall in brain tissue ATP may indicate a wider cerebral metabolic deficit possibly associated with reduced cerebral blood flow (CBF) in addition to hypoxia (Duffy *et al*, 1975). The rise in brain tissue ADP and AMP following a 30 minute occlusion period is consistent with the fall in ATP and therefore suggests that ATP stores could not be regenerated during the insult. In addition, a rise in ADP and AMP is known to stimulate phosphofructokinase and this may be one source of the sustained stimulation of anaerobic glycolysis (Siesjö, 1978). The continued anaerobic glycolytic flux associated with a thirty minute occlusion was not sufficient to maintain ATP supplies and, since ATP is necessary for a variety of metabolic reactions as well as maintenance of ATP-ase governed ionic gradients, it is reasonable to assume that the occlusion was associated with disruption of cerebral metabolism (Siesjö, 1989; Vannucci, 1990).

Incorporation of a twenty minute recovery period was associated with some metabolic changes but few metabolites recovered to control levels, particularly after a thirty minute occlusion. Brain tissue lactate rose significantly during the recovery period which could indicate continued elevation of anaerobic glycolytic flux above that in control rats but it may also be related to enhanced uptake of lactate from the restored maternal blood supply to the foetus (Cremer, 1982). A rise in brain tissue glucose also occurred during the recovery period which again is indicative of restoration of substrate supply from the uterine arteries. Following a perinatal hypoxic-ischaemic insult it is

believed that the uptake of glucose and lactate from blood to brain is enhanced by stimulation of the uptake carrier mechanism as well as by facilitated diffusion and these results are consistent with this view (Vannucci and Duffy, 1976; Cremer, 1982).

The lack of a significant effect of recovery period on hypoxic brain tissue ATP and PCr, especially noticeable after a 30 minute occlusion period (see Tables 3.12 and 31.6), in addition to the continued rise in lactate may indicate a persistent failure of cerebral oxidative metabolism during the recovery period.

The changes in brain tissue metabolites measured in hypoxic rat brains during the first three hours of postnatal life are indicative of a complete recovery of cerebral metabolic function. During the first 60 minutes of post-natal life hypoxic rat brain lactate declined rapidly but brain glucose increased which is consistent with the observation that lactate is oxidised in preference to glucose during recovery from hypoxia in the perinatal rat brain (Vannucci and Duffy, 1976). In control rat brains a post-natal rise in glucose and decline in lactate also indicated a preference for lactate as the substrate for cerebral oxidative metabolism (Hawkins *et al*, 1971).

The recovery of cerebral metabolic function appeared to be maximal between one and 60 minutes after delivery such that the hypoxic rat brain ATP and PCr rose to levels similar to control rats during this time. The postnatal rise in brain tissue ATP and PCr was most dramatic for those rats which had been exposed to a 30 minute occlusion period so, although this occlusion caused the greatest cerebral metabolic disruption, the effects were not particularly long lasting and all surviving rats appeared to recover normal cerebral metabolic function.

The decline in sham-operated and control rat brain tissue PCr during the first minute of life was taken to indicate a transient hypoxic insult and has been observed in other rats delivered by Caesarian section (Vannucci and Duffy, 1979). The slight delay in the onset of spontaneous gasping was evident in all rats exposed to the anaesthetic and alterations in brain tissue metabolites measured in the sham-operated rats suggest an influence of isoflurane anaesthesia on the cerebral metabolic rate. All measured brain metabolites were increased in sham-operated rats and a rise in ATP and PCr,

particularly, may indicate a depression in cerebral metabolism which is consistent with previous observations of reduced cerebral oxygen consumption during isoflurane anaesthesia (Eger, 1981; Cucchiara and Michenfelder, 1990). The nitrous oxide/oxygen mixture through which the anaesthetic was inspired is not believed to have an effect on CBF or cerebral metabolic rate in the rat (Cucchiara and Michenfelder, 1990). There were no significant differences between metabolites measured in control rat brains from the 10, 20 or 30 minute surgical procedures which indicates that the duration of anaesthesia had no direct effect on foetal cerebral metabolism during the surgery.

The lack of significant differences between the sham-operated rats and the control rats might be taken to indicate that the surgical procedure itself had no direct influence on the foetal rat brain metabolism. This factor also demonstrates that the occlusion of vessels on the adjacent uterine horn had no significant influence on the measured control foetal rat brain metabolites.

3.2 A HISTOLOGICAL SURVEY OF THE RAT BRAIN FOLLOWING EXPOSURE TO PERINATAL HYPOXIA

3.2.1 The Neonatal Rat Brain

The very high water content, large ventricles and lack of myelin of perinatal rat brain caused many problems when attempting to achieve a consistent, high quality of sections and staining for histological analysis. Accuracy of perfusion fixation was the main limiting factor and the end result was a very low number of brains, taken from rats at 4 hours of age, with which we were satisfied that we had achieved the best histology. However, when the neonatal rats brains were successfully perfusion fixed at 4 hours of age, it was not possible to differentiate between the control and the hypoxic rat brains whatever the duration of the occlusion period.

Neonatal rat brains that were perfusion fixed on pnd3 were around twice the size of the 4 hour old rat brains and, although there was still no myelin, the development of the regional structures had progressed significantly. The gross morphology of control and hypoxic rat brains was similar and there was no evidence of swelling, haemorrhage or infarct in the brains of rats exposed to the 30 minute *in utero* occlusion. At the light microscope level there was no evidence of selective neuronal necrosis in anterior regions, in the striatal region or in the neocortex of the hypoxic rat brain. Photomicrographs 3.1 and 3.2 show the hippocampal region of a 3 day old control and a hypoxic rat brain respectively. In both the control and the hypoxic rat brains a high number of very darkly stained neurons can be seen. These neurons may be the victims of naturally occurring cell death (Clarke, 1985; Oppenheim, 1991). Photomicrographs 3.3 and 3.4 show the CA1 regions of the control and the hypoxic rat brains. The density of live cells in each rat brain appeared to be similar. The density of live cells in the CA3 regions was similar for the two groups (photomicrographs 3.5 and 3.6) but in 4 out of 5 brains assessed there appeared to be around 5-10% more dark stained neurones in the hypoxic CA3 region than in the control CA3 region. This increase in dark stained neurons may however, be an artefact due to inadequate fixation (Cammermeyer, 1978).

Further examples of control and hypoxic rat brains at pnd3 can be found in Appendix 1.

The hind brain regions, particularly around the pons, were similarly affected with a minimal increase in the number of darkly stained cells in the hypoxic brains. Photomicrographs 3.7 - 3.10 demonstrate the most noticeable difference between control and hypoxic rat brains; in several regions of the brain, particularly the hind brain, there was a small degree of neovascularisation. The photomicrographs demonstrate the prominent staining, using haematoxylin and eosin, of these new blood vessels. Other regions affected included the neocortex and the striatum but to a lesser degree than the hind brain regions. This neovascularisation was found in all observed brains perfused at 3 days of age after exposure to a 30 minute *in utero* occlusion.

For those brains perfusion fixed at pnd14 and pnd21 there were no significant differences between control and hypoxic rats at any level in the brain and therefore no photomicrographs are shown.

3.2.2 The Adult Rat Brains

Following completion of the behavioural studies all of the adult rats were perfusion fixed and then sectioned coronally at 30μm using a cryostat. At this thickness accurate cell counts could not be made and so the aim was to observe the gross appearence of the control and hypoxic rat brains. In this way it would be possible to record any areas of extensive cellular necrosis but subtle alterations in cell density would not be detected.

Photomicrographs 3.11 and 3.12 respectively show the hippocampal region of a control and a hypoxic rat brain. There was no apparent difference at gross or light microscopic levels between the two groups of rats and, as photomicrographs 3.13 and 3.14 show, the density of cells in the CA1 region appeared to be similar for both groups. However, when the width of the CA1 region was measured using an image analyser there was a significant 3-6% reduction in the width of the hypoxic brain CA1 region in comparison with the control CA1 ($p < 0.05$; see table 3.19). Anova showed that the

difference between the width of the control CA1 and that of the hypoxic CA1 region was significant ($F(1,68) = 6.18$, $p = 0.015$) and interestingly there was also a significant difference between left and right sides ($F(1,68) = 7.31$, $p = 0.009$) which was seen in both control and hypoxic CA1 regions. There was however, no significant difference between the widths of the dentate gyrus in the two groups ($F(1,44) = 2.1$, $p = 0.16$) which might suggest that the reduction in hypoxic brain CA1 width reflects some alteration in cell density, cell size or dendritic outgrowth after exposure to the perinatal trauma. The difference between left and right CA1 regions cannot easily be explained but there was no significant differnce between sides in the dentate gyrus of control or hypoxic brains ($F(1,44) = 0.01$, $p = 0.91$). The fact that the difference between the sides was evident in both control and hypoxic CA1 regions suggests that the difference was not due to exposure to the perinatal trauma.

Photomicrographs 3.15 - 3.16 demonstrate that the densities of cells throughout both control and hypoxic adult rat brains appeared to be similar but no detailed area or cell density measurements were made.

TABLE 3.19 MEAN WIDTH MEASUREMENTS (mm) OF CA1 AND DENTATE GYRUS IN ADULT HIPPOCAMPUS

REGION	CONTROL			HYPOXIC		
	LEFT	RIGHT	n	LEFT	RIGHT	n
CA1	7.66×10^{-2} $\pm 0.14 \times 10^{-2}$	7.18×10^{-2} $\pm 0.13 \times 10^{-2}$	36	7.21×10^{-2} $\pm 0.17 \times 10^{-2}$	6.91×10^{-2} $\pm 0.14 \times 10^{-2}$	36
DENATE GYRUS	9.56×10^{-2} $\pm 0.33 \times 10^{-2}$	9.34×10^{-2} $\pm 0.35 \times 10^{-2}$	24	9.81×10^{-2} $\pm 0.42 \times 10^{-2}$	10.11×10^{-2} $\pm 0.29 \times 10^{-2}$	24

3.2.3 Immunohistochemical Staining of Neonatal Rat Brains

The GFAP staining of rat brains which had been perfusion fixed at pnd3 or pnd7 produced poor sections. Although any expected astrocytic reaction to the hypoxic insult would be evident at 3 days and particularly at 7 days of age those sections which could be observed microscopically showed no difference between control and hypoxic rats.

The quality of staining improved in sections taken from rat brains which had been fixed at pnd14 or pnd21. Photomicrographs 3.17 and 3.18 show the hippocampal region of 21 day old control and hypoxic rat brains respectively but no differences (i.e. no glial scarring) were apparent in the hypoxic rat brain, which is in agreement with a lack of astrocytic reaction 3 and 7 days after the insult.

3.2.4. Discussion

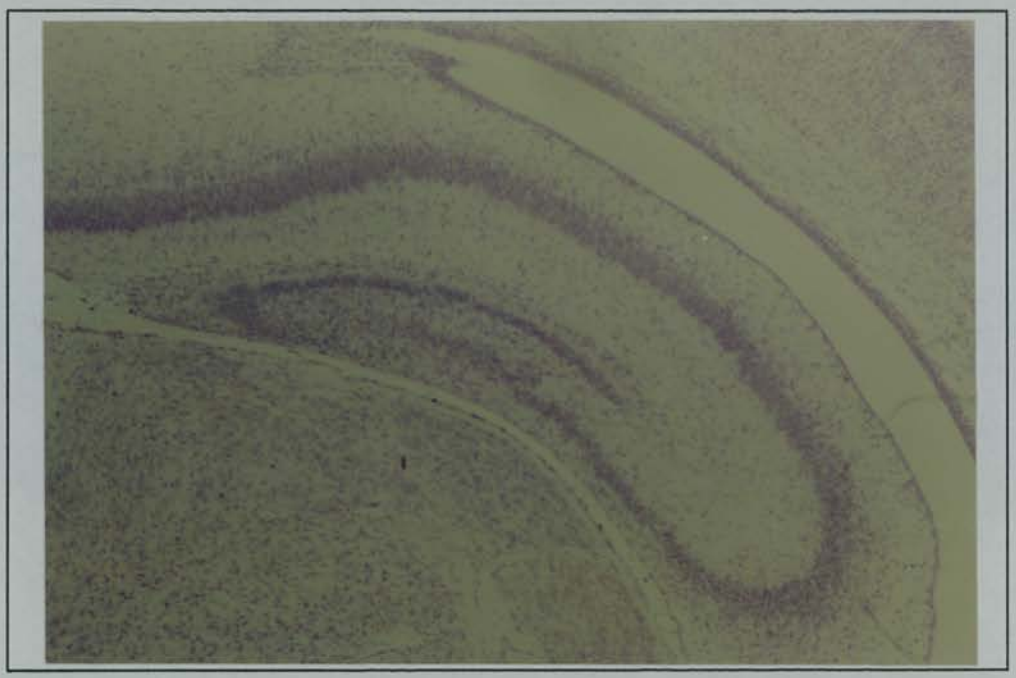
It was somewhat surprising that the considerable disturbance of cerebral metabolism reported in the previous section did not seem to produce any obvious regions of widespread neuronal necrosis or neuroanatomical reorganisation. The brain sections investigated were not subjected to detailed densiometric analysis but visual inspection of the adult hypoxic rat brains would suggest that those discreet changes identified at pnd3 were not associated with any great degree of irreversible neuronal necrosis. The use of width measurements did indicate some alteration in the hypoxic CA1 region but this does not neccesarily reflect neuronal necrosis.

It has been suggested that the darkly stained cells located in the CA3 region of the 3 day old rat hippocampus were victims of naturally occurring cell death. The pathological profile of naturally occurring cell death is frequently described as apoptosis and the cellular contraction characteristic of apoptosis is not dissimilar to the pyknotic profile of the cells identified in both control and hypoxic rat brains (Wyllie, 1981; Oppenheim, 1991). Postnatal naturally occurring cell death has been identified in certain, but not all, hippocampal regions (Janowsky *et al*, 1983; Boss *et al*, 1987; Oppenheim, 1991) so it is feasible that the darkly stained cells do correspond to dying neurons but the possibility that the dark cells are due to histological artefact cannot be ignored (Cammermeyer, 1978). It is, however, of interest to note that pyknotic cells were not found in the CA1 region where it is believed that naturally occurring cell death does not occur (Boss *et al*, 1987).

Ischaemia and the associated reduction in oxygen supply to tissues has been found to stimulate neovascularisation in an attempt to compensate for the oxygen deprivation (Adair *et al*, 1990; Schweiki *et al*, 1992; Nakada and Dyck, 1986). It is believed that hypoxia is responsible for stimulation of endothelial cells, located in the microvasculature, which produce vascular endothelial growth factor and thereby promote angiogenesis (Knighton *et al*, 1983; Schweiki *et al*, 1992). Neovascularisation has also been shown to occur in response to excitotoxic kainic acid lesions of the rat striatum (Shigematsu *et al*, 1989). The compensatory nature of neovascularisation may be responsible for the appearance of prominent staining of microvessels in the 3 day old hypoxic rats and since angiogenesis can occur prior to gliosis extensive injury need not accompany this cerebral tissue response to hypoxia (Shigematsu *et al*, 1989).

The lack of significant widespead neuronal necrosis associated with this model of perinatal hypoxic-ischaemia is in conflict with results obtained from other methods of induction of perinatal hypoxic-ischaemia/anoxia. The induction of total asphyxia in newly-delivered guinea-pig and monkey foetuses is associated with subcortical neuronal necrosis in areas such as the thalamus, globus pallidus and brain stem (Windle and Becker, 1943; Ranck and Windle, 1959; Windle, 1966; Myers, 1979). Partial asphyxia in the foetus and neonate is associated with more widespread damage which extends into cortical regions and can be accompanied by brain swelling and haemorrhage (Bailey and Windle, 1959; Myers, 1972). The small but significant reduction in hypoxic CA1 width might not reflect neuronal necrosis but if it does then it is consistent with many studies of perinatal hypoxic-ischaemia. A recent model of perinatal asphyxia in the rat, by submersion of the uterus, has been found to reduce neuronal density in CA1 and CA3 regions of the hippocampal but to increase tyrosine hydroxylase staining in the substantia nigra (Bjelke *et al*, 1991). Induction of anoxia in the early postnatal period also reduced CA1 cell density although no other brain regions are affected (Dell'Anna, 1991).

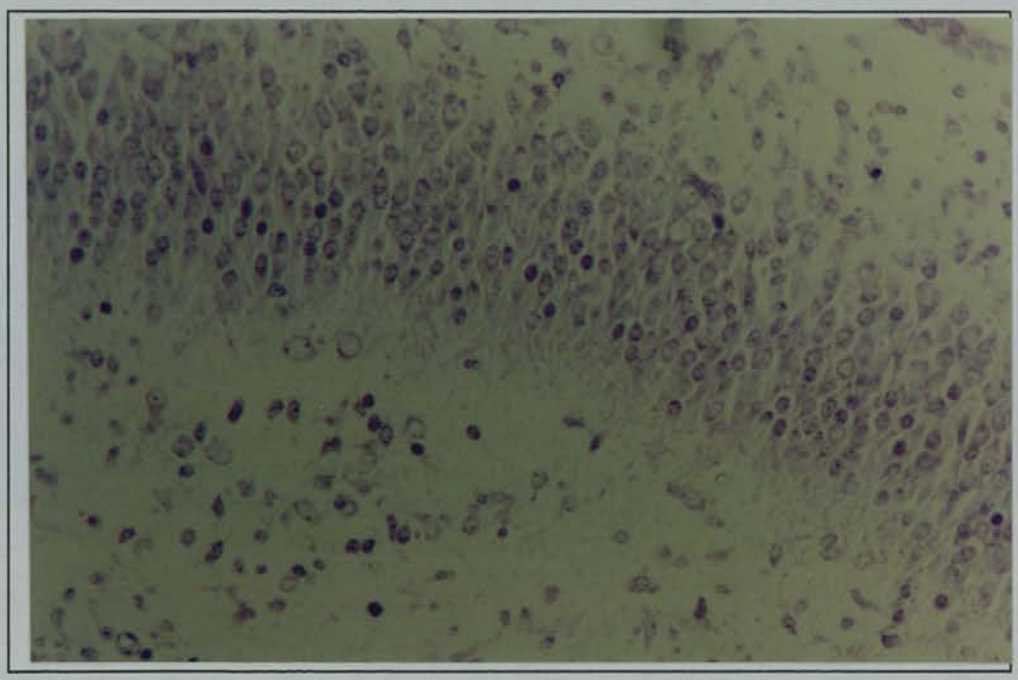
It would appear that the model reported here is in direct conflict with those that produce extensive neuronal necrosis. However, the pattern of damage produced in other models is hugely variant and entirely dependent upon the method of induction of hypoxic-ischaemia and the duration of the insult. Hicks and co-workers (1962) reported that early postnatal exposure to anoxia caused no neuronal death and only disturbed process formation in the outer cortical layers such that they were thinner than in control animals. Bailey and Windle (1959) state that the long-term survival of guinea-pigs exposed to perinatal asphyxia was associated with limited neuropathological change. Similarly, this model of uteroplacental vessel occlusion would appear to cause discreet changes in the early postnatal period but in those rats which survive to adulthood it might be reasonable to suppose compensatory mechanisms and/or a high level of resistance prevent extensive long-term neuropathological damage. The alteration in CA1 width merits further investigation of a more detailed nature with thinner sections and in this way the question of whether or not the reduction in width is due to neuronal necrosis can be answered. In addition it is possible that subtle changes throughout the hypoxic rat brains could be detected with a more extensive and detailed examination of cell density, laminar thickness and process formation.



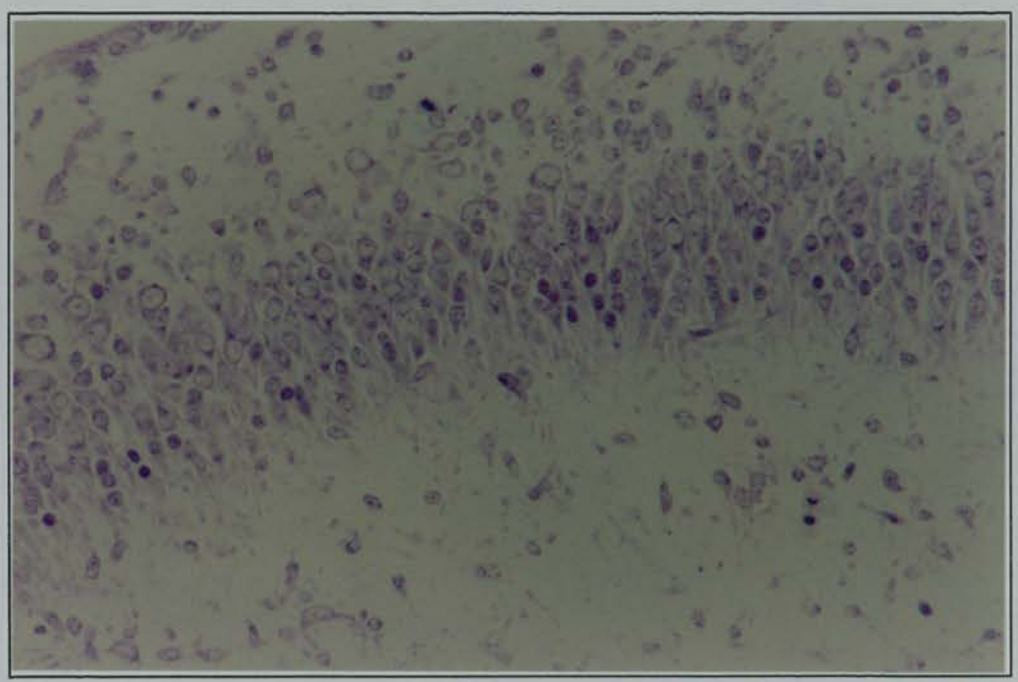
3.1

3.2

Photomicrographs 3.1 and 3.2
The photographs show the hippocampal region of a control rat brain (3.1) and a hypoxic rat brain (3.2) which had been perfusion fixed with FAM on pnd3. Following fixation the brains were dehydrated, cleared in xylene and embedded in molten paraffin wax. Coronal sections were taken at 6μm and then stained with cresyl violet. Magnification = 40x

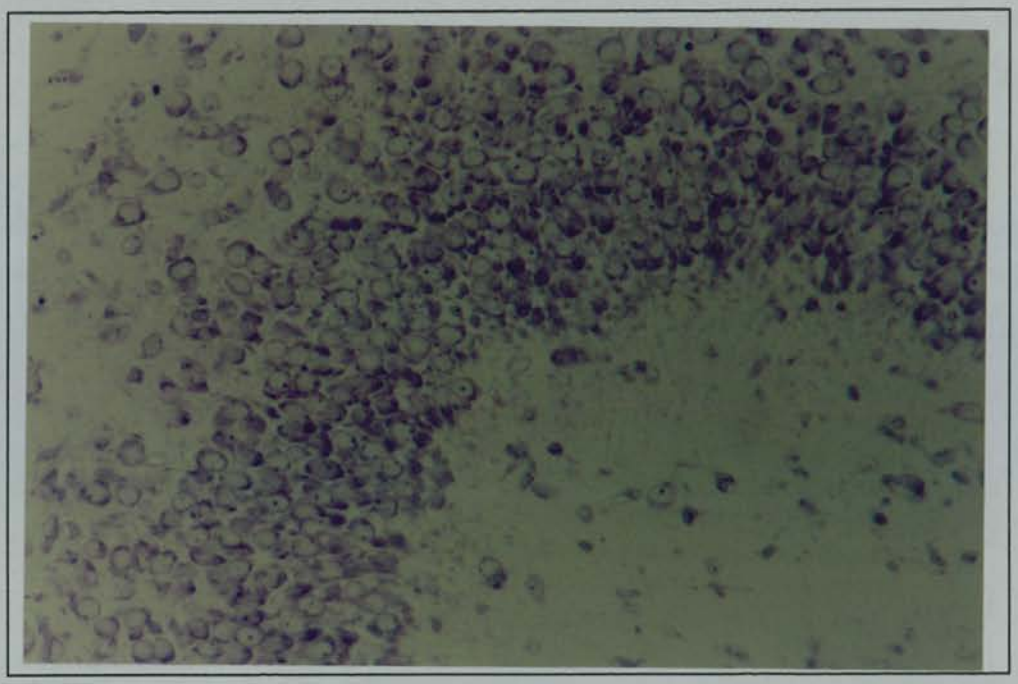


3.3

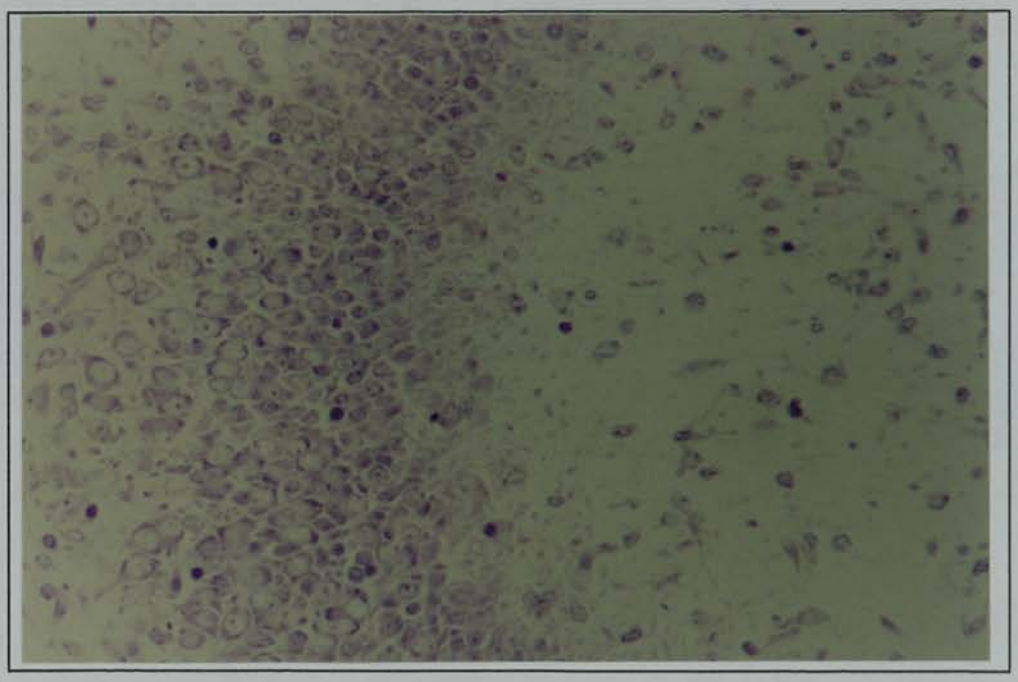


3.4

Photomicrographs 3.3 and 3.4
The CA1 region of the hippocampus in a control rat brain (3.3) and a hypoxic rat brain (3.4) at 3 days of age is shown. See 3.1 for details of staining procedure. The density of cells in both sections appears to be similar. Magnification = 200x.

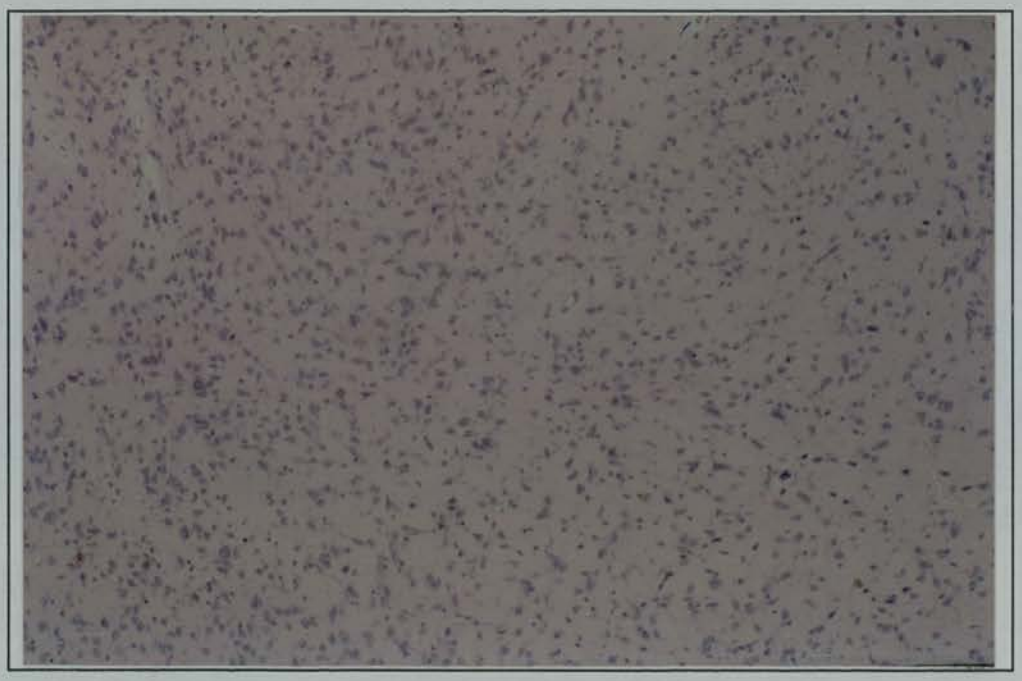


3.5

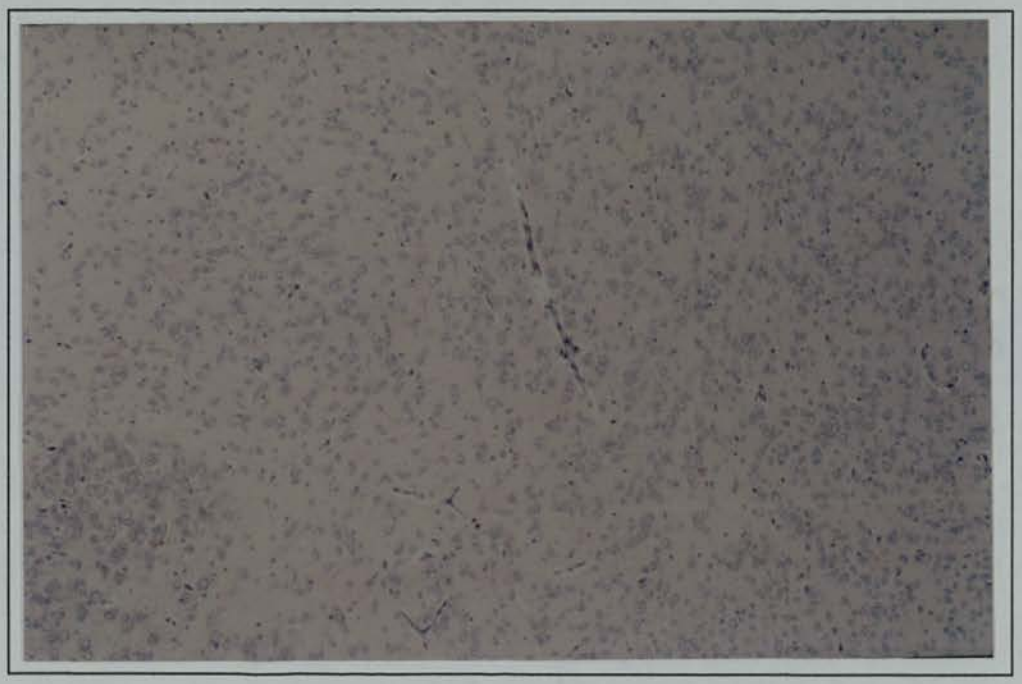


3.6

Photomicrographs 3.5 and 3.6
The photomicrographs shows the hippocampal CA3 region of a 3 day old control rat brain (3.5) and a hypoxic rat brain (3.6). See 3.1 for details of processing. If the photomicrograph is compared with 3.5 a small increase in the number of darkly stained neurons is noted. Magnification = 200x.

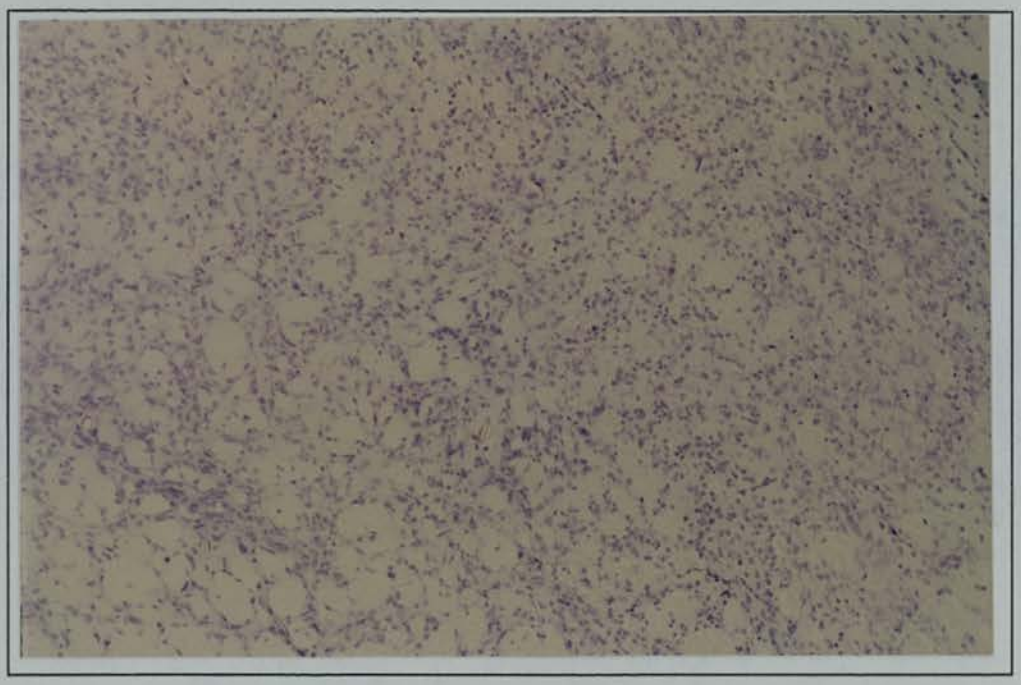


3.7



3.8

Photomicrographs 3.7 and 3.8
The photomicrographs show the hindbrain regions of 3 day old control and hypoxic rat brains (3.7 = control rat and 3.8 = hypoxic rat). The brains were processed as described for photomicrograph 3.1 but were stained with haematoxylin and eosin. The hypoxic rat brain has number of new capillaries not seen in the control rat brain. Magnification = 75x.

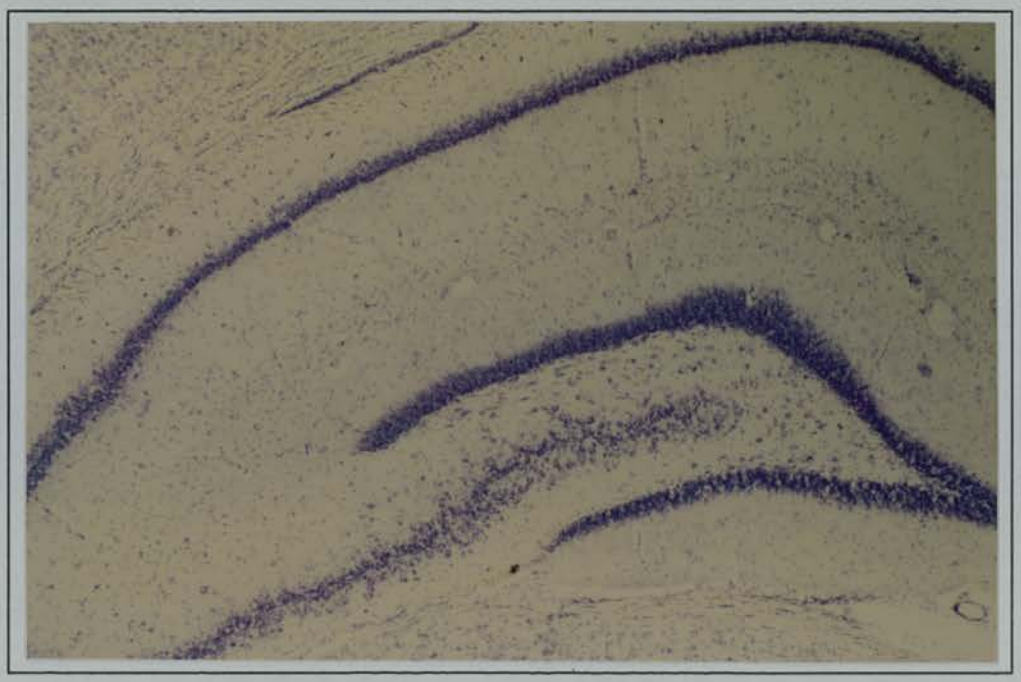


3.9

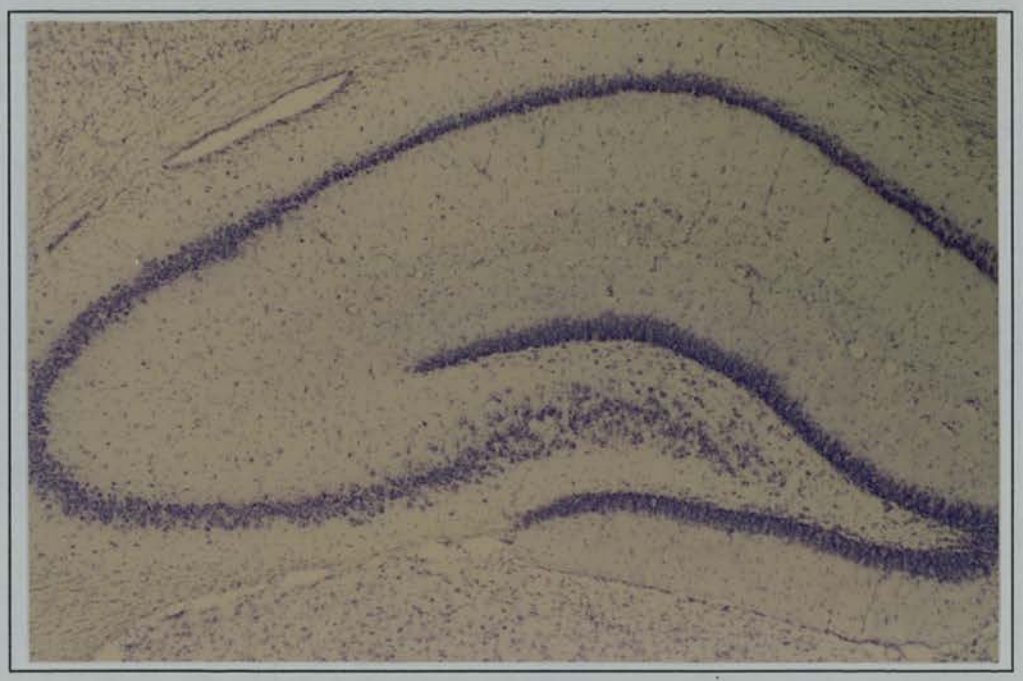
3.10

Photomicrographs 3.9 and 3.10

These photomicrographs are taken from the striatal regions of 3 day old control (3.9) and hypoxic (3.10) rat brains. As also shown in hind brain (3.8) there is a small degree of prominent staining of small blood vessels in the hypoxic striatum but to a much lesser extent than the hind brain. Magnification = 75x.

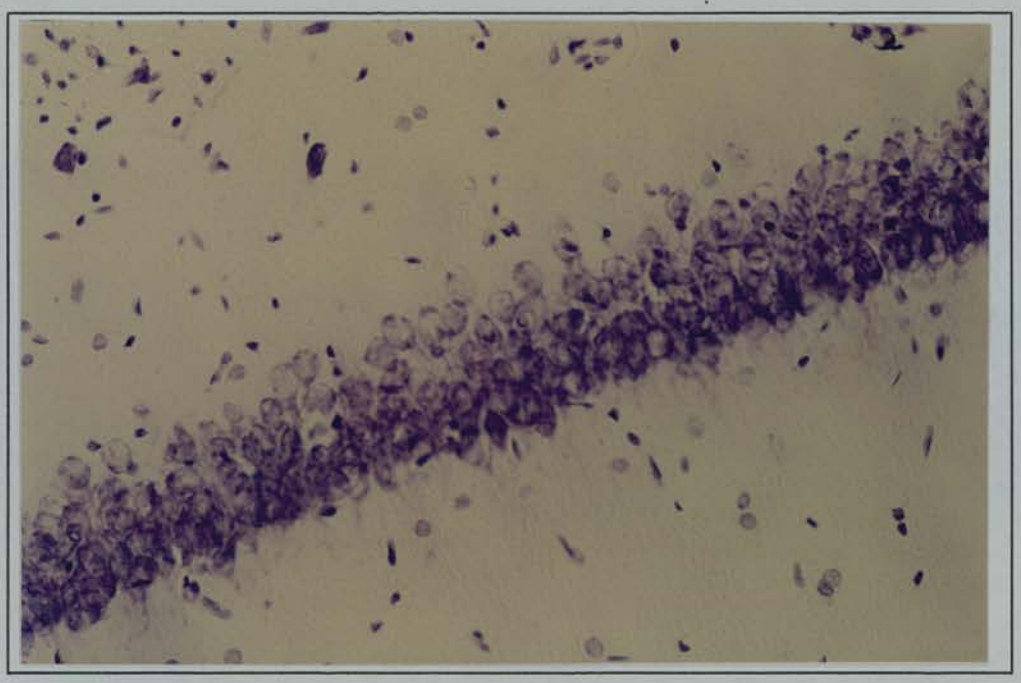


3.11



3.12

Photomicrographs 3.11 and 3.12
The photomicrographs depict the hippocampal region of an adult control rat brain (3.11) and of a hypoxic rat brain (3.12). The rats were perfusion fixed with 10% buffered formalin and the brains were sectioned coronally at 30μm and then stained using cresyl violet. Magnification = 31.25x.



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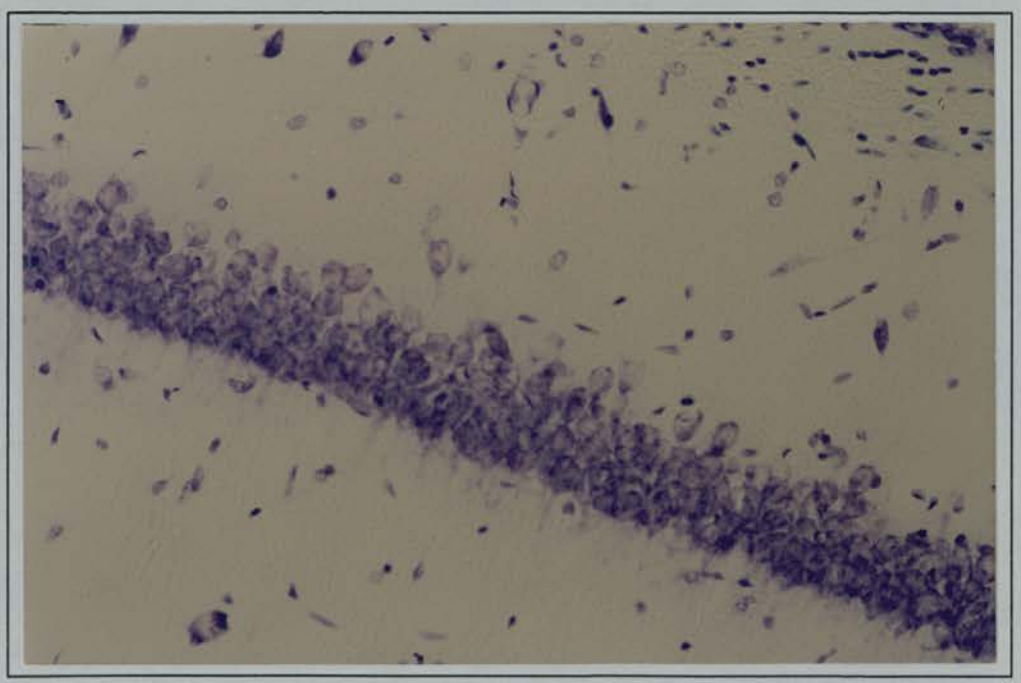
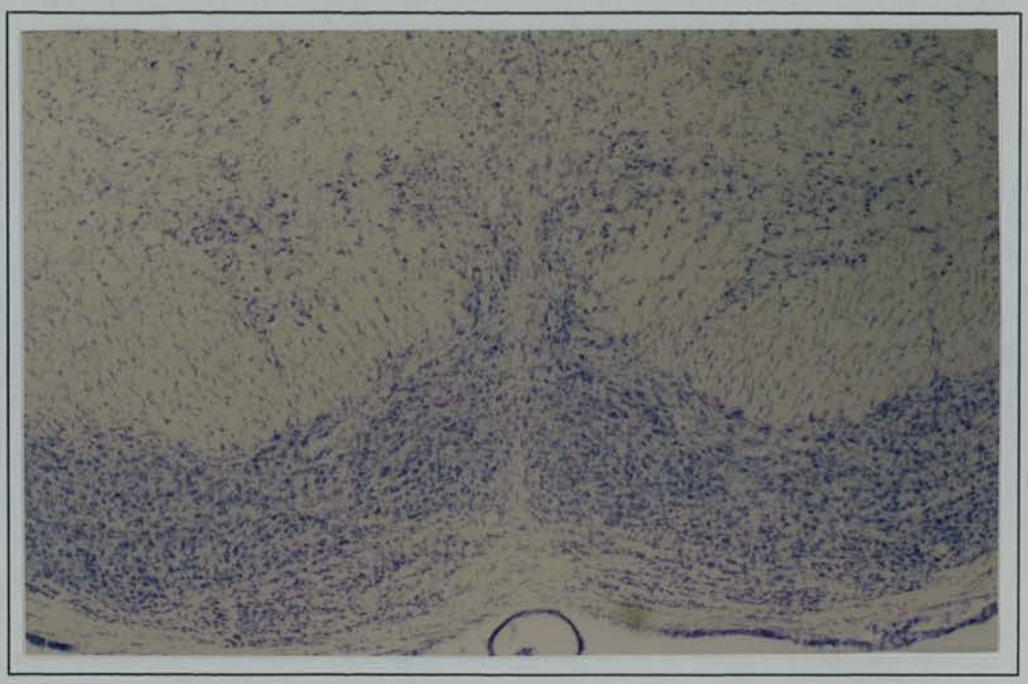


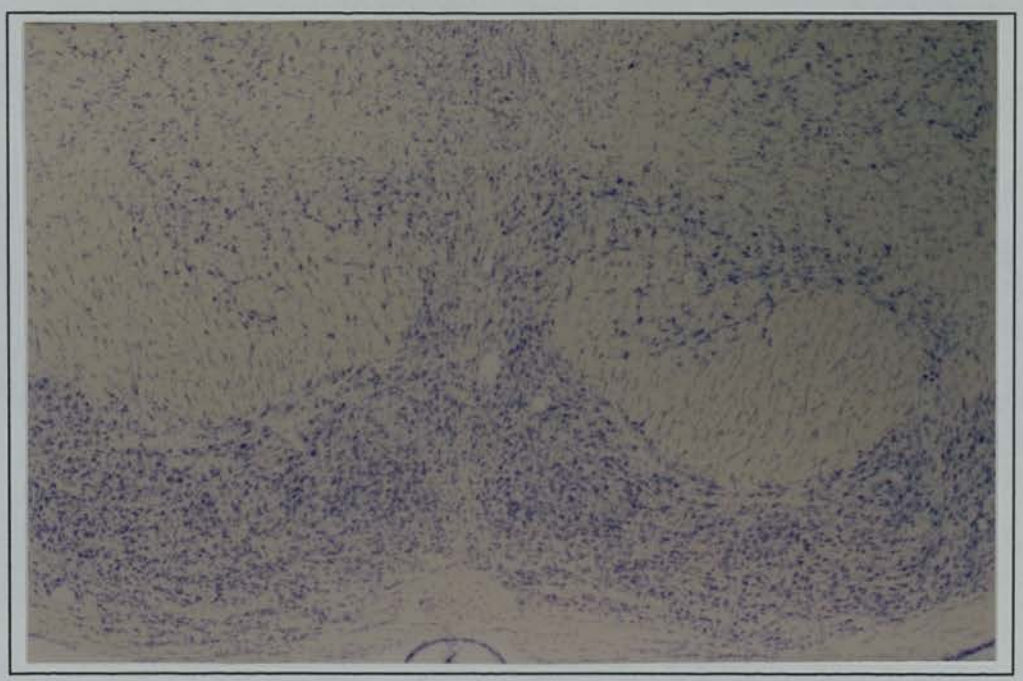
3.14

Photomicrographs 3.13 and 3.14

The hippocampal CA1 regions of the adult rat brains shown in 3.11 and 3.12 are represented in 3.13 (control) and 3.14 (hypoxic). At 30μm cell counting was not possible but when the widths of the CA1 regions were compared there was a significant 3-6 % reduction in hypoxic CA1 width ($p < 0.05$). Magnification = 75x.

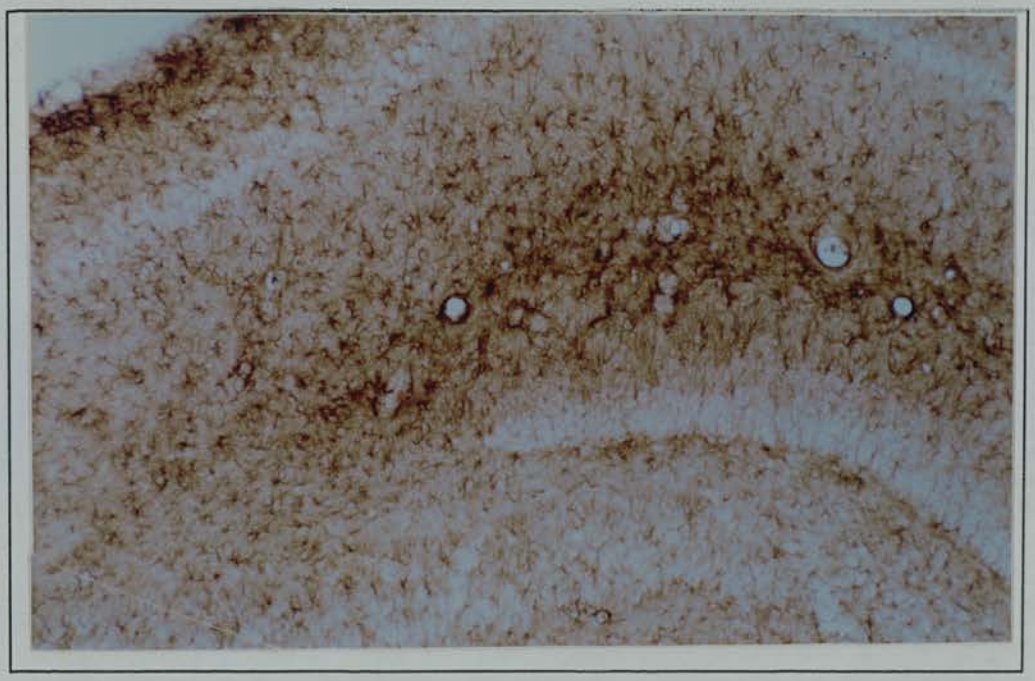


3.15

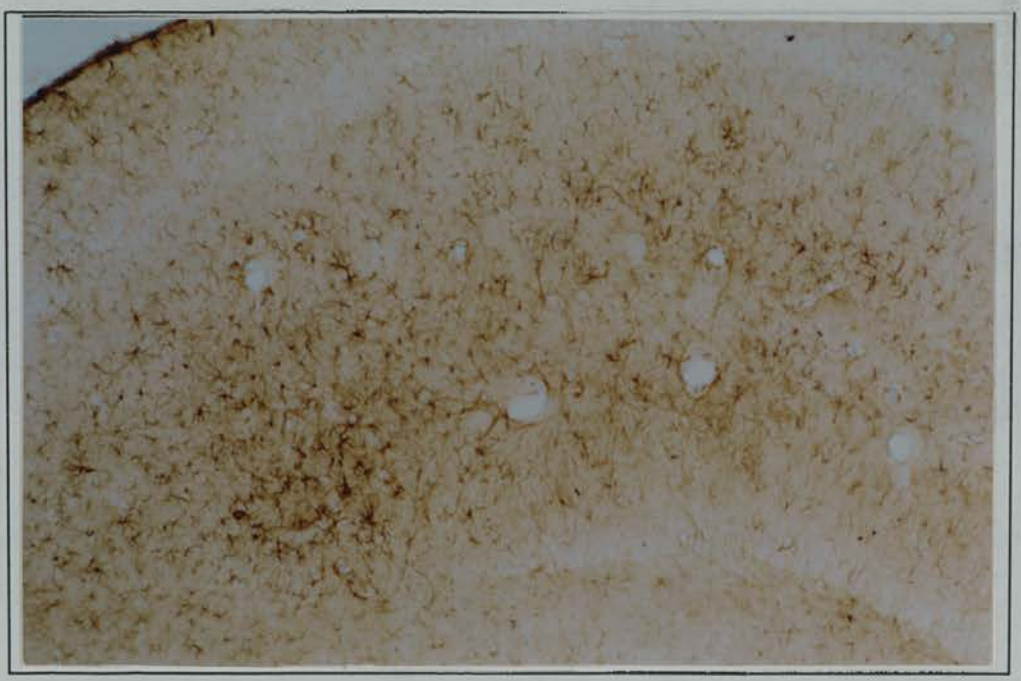


3.16

Photomicrographs 3.15 and 3.16
The photomicrographs depict the pons region of an adult control rat brain (3.15) and an adult hypoxic rat brain (3.16). For details of processing of the brains see 3.11 and 3.12. Magnification = 31.25x.



3.17



3.18

Photomicrographs 3.17 and 3.18

The GFAP immunohistochemical stain was used on 21 day old rat brains which had been perfusion fixed with 4% paraformaldehyde + 0.05% gluteraldehyde. The avidin-peroxidase technique was used to develop the stain.

Photomicrograph 3.17 depicts the hippocampal region of a control rat brain and photomicrograph 3.18 depicts the hippocampus of a hypoxic rat brain. Magnification = 31.25x.

3.3 POST-NATAL BEHAVIOUR AND DEVELOPMENT OF RATS EXPOSED TO A HYPOXIC INSULT IN UTERO

3.3.1 Post-operative Behaviour and Development

a) Immediately after delivery

After completion of each surgical procedure the foetal rats were delivered by Caesarian section. The majority of rats were gasping within the amniotic sacs therefore delivery had to be completed within 30-60 seconds. All of the foetal rats that survived were gasping on delivery and any rats which were pale and not gasping did not survive. Typically the hypoxic rats were paler than the control rats and, especially after a 20 or 30 minute occlusion period, fewer were gasping. When the foetal rats were delivered before a 20 minute recovery period (without anaesthesia) it was particularly difficult to resuscitate the control pups.

b) 5-10 minutes after delivery

Within 5-10 minutes after delivery the surviving neonatal rats had brought up a great deal of mucus which was cleared away using absorbent tissue. Breathing at this stage was achieved through rhythmic and frequent gasping. All of the rats were ataxic at this stage with all movement restricted to the vigorous breathing effort.

c) 10-20 minutes after delivery

10-20 minutes after delivery the gasping effort was replaced by diaphragmatic nasal breathing. Many neonatal rats, particularly the hypoxic rats, had to be manually stimulated during the transition from gasping to normal breathing because of frequent periods of apnoea. It can be supposed that this function would normally be served by an attentive dam whilst washing the neonates.

d) 20-120 minutes after delivery

After 20 minutes there was a 5-10 minute period of respiratory depression in 30-50% of neonates. This took the form of slower, less frequent breathing and, sometimes, apnoea. Gentle manual stimulation and turning usually restored the breathing effort.

During the first 20 minutes in the incubator there were no visible differences between control and hypoxic rats. It was not always the case that the control rats established regular breathing patterns before the hypoxic rats, particularly in those experiments where there was no 20 minute recovery period. Occasional convulsive activity was noted in 10-20% of hypoxic rats. The activity involved slight twitching and sometimes vigorous whole body convulsions but only occurred in those rats exposed to a 30 minute occlusion period.

20-30 minutes after delivery the neonatal rats began to exhibit signs of independent movement. The larger rats were able to roll in to an upright position and move their heads from side to side. Over the next 30-60 minutes the neonatal rats became more active with a great deal of rolling from side to side and some dragging/crawling movements around the incubator. 50-70% of the hypoxic rats which had been exposed to a 20 or 30 minute occlusion period were slower to develop independent movement. The level of activity in these hypoxic rats was less than their littermate controls for around 30-45 minutes.

120 minutes after delivery there was rarely any visible difference between control and hypoxic rats.

3.3.2 The Level of Mortality of Foetal Rats Following *in utero* Hypoxic Episodes

Table 3.20 summarises the mortality figures for each experimental procedure. All rats that could not be resuscitated within 10 minutes after delivery were included in these data.

The data for sham-operated rats suggest a significant effect of anaesthesia on mortality. None of the normal rats, which were not exposed to anaesthetic, died whereas over 50% of those rats exposed to 25 minutes of anaesthesia died. Inclusion of a 20 minute recovery period after anaesthesia reduced the level of mortality in sham operated rats to 5%. The severity of the effects of anaesthesia can also be seen in the data for control rats. As the period of anaesthesia increased a greater proportion of the

control rats could not be resuscitated. The recovery period of 20 minutes without anaesthesia always improved the control rats chance of survival whatever the initial period of anaesthesia.

Comparison of the mortality figures for control and hypoxic rats suggested that a hypoxic episode severely restricted a rat's chance of survival. Rats exposed to hypoxia without a recovery period were less likely to die than their littermate controls although this difference lessened with the longer occlusion period. Rats exposed to a recovery period after the hypoxic insult were more likely to die than their littermate controls (7 times more likely after a 30 minute occlusion period). The 20 minute recovery period had little effect on the survival of hypoxic rats. The length of the occlusion period was important because rats exposed to a 30 minute occlusion period were 3 times more likely to die than those exposed to a 10 minute occlusion period.

The duration of anaesthesia and presence of a recovery period were the important factors influencing a control rat's chance of survival. The length of the occlusion period was the main factor influencing a hypoxic rats chance of survival. The effect of the anaesthetic on hypoxic rats is unlikely to be significant because many rats died following an occlusion without a recovery period, which restricted the supply of anaesthetic to the foetal rats.

3.3.3 Mortality Figures for the Cross-Fostering Procedure

In order to make assessments of the hypoxic rats during post-natal development the hypoxic neonates and their littermate controls were cross-fostered onto lactating Cob-Wistar dams. Table 3.21 shows a summary of all the attempts at cross-fostering. The majority of hypoxic rats had been exposed to a 30 minute occlusion period with a 20 minute recovery plus 2 hours in the incubator before being cross-fostered.

42% of cross-fostered control rats died which suggested that the success of the procedure was limited. Many factors may have influenced the control rats' chances of survival such as a previous exposure to anaesthetic and the Cob-Wistar dam's failure to accept foreign offspring.

TABLE 3.20 MORTALITY FIGURES FOR ALL EXPERIMENTAL CONDITIONS

EXPERIMENTAL PROCEDURE	CONTROL RATS			HYPOXIC RATS		
	NO. RATS	NUMBER DIED	% OF TOTAL	TOTAL NO. RATS	NUMBER DIED	% OF TOTAL
NON-OPERATED	88	0	0%			
SHAM + 0 mins recovery	61	32	52.5%			
SHAM + 20 mins recovery	71	4	5.6%			
10 mins + 0 mins recovery	43	20	46.5%	34	10	29.4%
10 mins + 20 mins recovery	54	2	3.7%	51	9	17.7%
20 mins + 0 mins recovery	57	35	61.4%	62	31	50.0%
20 mins + 20 mins recovery	88	9	10.2%	95	48	50.5%
30 mins + 0 mins recovery	92	61	66.3%	89	55	61.8%
30 mins + 20 mins recovery	299	25	8.4%	324	196	60.5%

The table shows the number of rats which could not be resuscitated after each experimental procedure had been completed. For the sham procedures 'non-operated' represents rats which were not exposed to anaesthetic whereas the other sham procedures resulted in the foetal rats being exposed to a period of 25 minutes anaesthesia.

TABLE 3.21 MORTALITY FIGURES FOR ALL NEONATAL RATS PLACED WITH COB-WISTAR FOSTER DAMS

GROUP	TOTAL NUMBER CROSS-FOSTERED	TOTAL NUMBER DIED	TOTAL NUMBER DEAD 0-24 hrs	TOTAL NUMBER DEAD 24-48 hrs	TOTAL NUMBER DEAD > 48 hrs
Control Rat Pups	118	49 (41.5%)*	35 (71.4%)**	8 (16.3%)**	6 (12.2%)**
Hypoxic Rat Pups	93	55 (59.1%)*	40 (72.7%)**	9 (16.3%)**	6 (10.9%)**

* Figure expressed as a percentage of the total number of neonatal rats cross-fostered in that group.

** Figure expressed as a percentage of the total number of cross-fostered rats which died.

The table shows the total number of neonatal rats which were placed with nursing Cob-Wistar foster dams, the number of rats which did not survive and the stages at which those rats died or were missing from the cage.

The exposure to hypoxia before birth significantly limited the neonate's chance of survival because the hypoxic rats were 1.5 times more likely to die than their littermate controls. In the majority of cases the rats died within 24 hours, 3 rats died 5 days after cross-fostering and one rat died 12 days later but the cause of death was undetermined. Exposure to hypoxia before birth had no significant effect on the time of death because a similar proportion of control and hypoxic rats died at similar times.

3.3.4 The Post-Natal Growth and Development of Rats which had been Exposed to an *in utero* Hypoxic Insult

All of the following data were recorded from the same two groups of rats. The hypoxic rats (n = 8) were exposed to a 30 minute occlusion period followed by a 20 minute recovery period and 2 hours after delivery were cross-fostered onto nursing Cob-Wistar dams. The control rats (n = 14) were the littermate controls of these hypoxic rats. Not all of the cross-fostered rats survived and to achieve the final group sizes ten litters were used including 34 control neonates and 25 hypoxic neonates. If the cross-fostered hypoxic rats died the matching littermate controls were taken out of the experiment.

There were 4 male rats and 4 female rats in the hypoxic group and 6 male rats and 8 female rats in the control group. Before being cross-fostered all rats were marked by an independent observer therefore all of the following observations were performed blind.

a) Growth Curve

The weight (grammes) and head-to-tail length (cm) of each cross-fostered rat was assessed from pnd0 until 3 months of age. Figure 3.11 illustrates the increase in body weight in proportion to length , the weight gain during development and the increase in head-to-tail length during growth for control and hypoxic rats. Unpaired t-tests were performed for each measurement but at no stage during development was there a significant difference between the two groups.

b) Early development (pnd0-42) of the neonatal rat

During the first two weeks of postnatal life there is a rapid progression in the physical development of the rat (Bolles and Woods, 1964; Altman and Sudarshan, 1975). The following observations are based on notes taken during the regular assessments of growth and behaviour of the two groups of rats.

i) Pre-Weaning Development

The gross physical development in the early neonatal period was very poor, the eyes were firmly closed, the ears tightly attached to the skull and the skin was without pigment or fur. Between pnd3-4 the ears began to lift and the black pigment began to show around the head and neck. By pnd5 the ears were lifted away from the skull. The first fur growth was detected between pnd7-9 and by pnd11 the whole skin was covered with a thin velvety layer of fur.

From pnd1-3 there was little activity and the pups slept a great deal. During the 3 minute observation period movement was restricted to lifting the head and moving it from side to side. The pups began crawling movements between pnd3-5. These movements involved lifting the head, moving it from side to side and then lowering the head and pushing forward using the front legs to move. Crawling behaviour was more prominent by pnd7 and between pnd7-9 the pups tended to crawl around the observation box more and more. By pnd11 the co-ordination of crawling behaviour had improved and the back legs were used a little. On pnd11 the majority of pups exhibited their first attempts at rearing up but their balance was poor and they always fell over.

By pnd11 the eyelids were visible and between pnd14-15 the eyes began to open. On pnd16 all of the rats' eyes were fully open and there was no difference between the time of opening for control and hypoxic rats. Between pnd15-17 the fur began to thicken and the non-pigmented areas became white rather than pink. There was a full, thick covering of fur on all pups by pnd19.

Between pnd13-15 considerable improvement was noted in the balance and co-ordination of the rats. When the rats crawled the abdomen was lifted from the floor of the cage and on pnd15 all rats began full quadruped walking. On the first day of quadruped walking the rats proceeded slowly, wobbled a lot and the steps were exaggerated and strained. By pnd17 the body was lowered during walking, the balance had improved and the pace quickened. As the rats' balance and co-ordination improved there was a noticeable increase in activity (pnd17-21) and the rats were running and turning frequently. Rearing up became a prominent feature of the 3 minute observations. At first the rats used the wall of the box for support, then by pnd19 they managed brief periods (3-5 seconds) without support and between pnd21-23 they began to use their tails for support and stayed upright for longer periods of time.

In the group cage the behaviour of each rat was different to that in isolation. Between pnd11-15 the rats began to groom each other paying most attention to the head and ears. As the communal grooming activity increased so too did play activity. On pnd13-15 gentle play was noticeable which simply involved the rats climbing on top of each other. Between pnd17-21 the play became more vigorous, games included running into each other, a little jumping and some biting particularly the ears and tails.

Although these observations were qualitative any delays in physical development, changes in rearing activity or delays in the onset of quadruped walking would have been noticed and no such delays or differences were noted which indicates that the pre-weaning development of hypoxic rats was similar to that of control rats.

ii) Post-Weaning development

After rats were weaned on pnd21 and on pnd22 there was little change in the activity of the pups but the type of activity changed a great deal. Initial attention to noise was noticed on pnd11-13 but on pnd22 the rats paid considerable attention to noises around the observation box. The rats appeared to be intensely curious of their surroundings

and wandered all over the box rather than around the perimeter as they had done previously. They stopped frequently to sniff the floor of the box but rarely looked around.

Between pnd21-25 the physical appearance of the rats changed little except for the head shape. Over this period the head lengthened and the nose became more pointed. By pnd25 the rats looked like small adult Lister-hooded rats except for the soft texture of the fur. Between pnd39-42 the fur took on the adult course, wiry texture.

Between pnd22-25 the rats began to groom in isolation which involved particular attention to the face and ears and also scratching of the sides. The rats tended to spend more time rearing up or sitting on their haunches. Sniffing was still the dominant activity and the rats paid particular attention to the corners of the observation box.

In the group cage the games became more aggressive and by pnd25 the first signs of submissive versus dominant play were evident. The rats began to jump a great deal and they also kicked up the shavings on the floor of the cage. The group play continued to be rough particularly amongst the larger litters and by pnd33-36 sexual activity was noticed. The male rats were sniffing the female rats and by pnd39 they were frequently jumping on top of the females.

When observed in isolation the rats began to slow down a little. The rats tended to sit and look around for long periods of time. From pnd28 onwards the rats paid considerable attention to the top of the observation box and some began attempting to jump out. Many rats would sit and watch the others in the group cage or the observer. By pnd42 the rats were so large that the observation box tended to restrict their movement so the majority spent their time looking for escape routes.

Regular observations ceased after pnd42 but at this time the physical development was near completion (excepting size), there was strong evidence of sexual ability and the play activity appeared similar to that in adult group cages. At no time during these six weeks of observation was any difference noted between the control and the hypoxic rats in their physical development or their behaviour.

c) Development of the mature-type righting reflex

The mature-type righting reflex involves a rapid, well-co-ordinated body twist which corrects the rat's body position from supine to upright. The righting reflex is not present in neonatal rats but develops in the first 2 weeks of life. Daily measurements of each rats righting attempts were made by placing the rat on its back and recording the movements and the time taken to achieve an upright position. Figure 3.12 illustrates the time taken by each rat, from pnd1-15, to right itself.

On pnd1 all rats were given a maximum of 120 seconds to roll into an upright position but over 50% were unable to right themselves. When the rats were placed on their backs they strained to bring the front and back limbs straight up achieving a U-bend shape. In this position the rats rocked from side to side and if righting was achieved it was more by luck than by skill. Between pnd2-4 the U-bend was gradually replaced with a body twist where the head and forelimbs turned one way and the hindlimbs were turned in the opposite direction. Righting was still achieved by rocking from side to side and frequently one or both hind limbs were trapped underneath the rat's body.

Between pnd6-7 the rats began to turn their heads before attempting to rock over into an upright position. The rats began to use their forelimbs to grip and support the turn between pnd6-8 and around pnd9 they also started to use their hindlimbs. Between pnd10-12 the body twist was no longer so exaggerated and the turn became quite well co-ordinated. As the rats began to use their limbs more efficiently and co-ordination improved the speed with which they righted fell to between 1-5 seconds.

Around pnd14-15 the righting reflex displayed all the characteristics of the mature reflex and the rats started to correct their position in mid-air. Between pnd16-20 the reflex matured to an imperceptible twist which could not be measured and therefore was given a score of zero. Both the control and hypoxic group of rats attained the mature type reflex at a similar time.

FIGURE 3.11 THE INCREASE IN BODY WEIGHT IN COMPARISON WITH HEAD-TO-TAIL LENGTH OF NEONATAL RATS FROM PND 0 TO 3 MONTHS OF AGE

The three curves illustrate the gross physical development of control and hypoxic rats. Measurements of body weight (gms) and head-to-tail length (cm) were made daily from pnd0 to pnd23 and then on pnd25, 28, 33, 36, 39, 42 and at 3 months old. The mean ± s.e.m. of some of these measurements were used in the construction of the growth curves.

The hypoxic rats had been exposed to a 30 minute occlusion period plus a 20 minute recovery period. The neonatal hypoxic rats and their littermate controls were cross-fostered onto nursing Cob Wistar dams. Unpaired t-tests showed no significant differences between the two groups but the males in each group were always at least 1.5 times larger than the female rats.

FIGURE 3.11

GROWTH CURVE

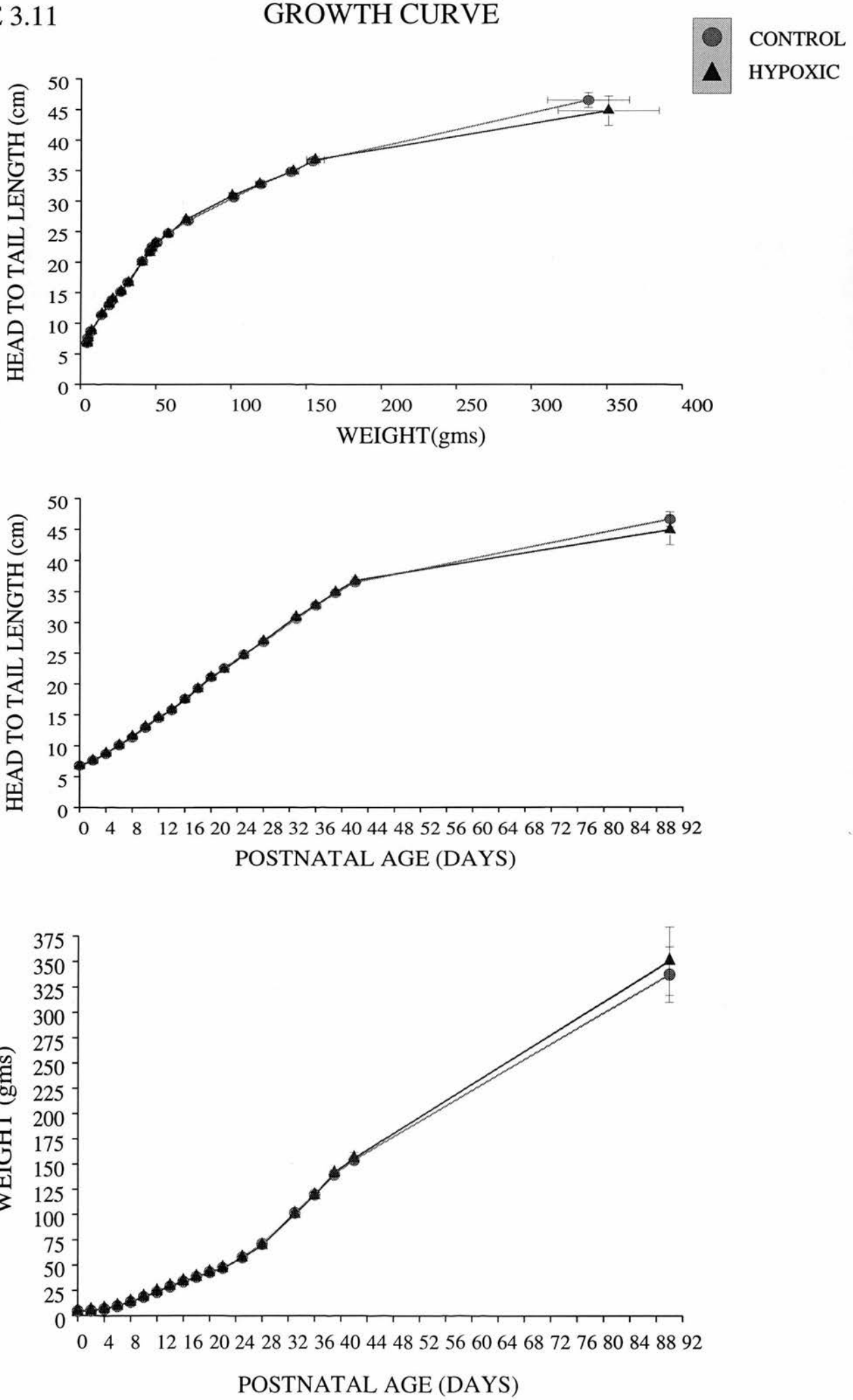


FIGURE 3.12 THE DEVELOPMENT OF THE MATURE-TYPE RIGHTING REFLEX IN NEONATAL RATS

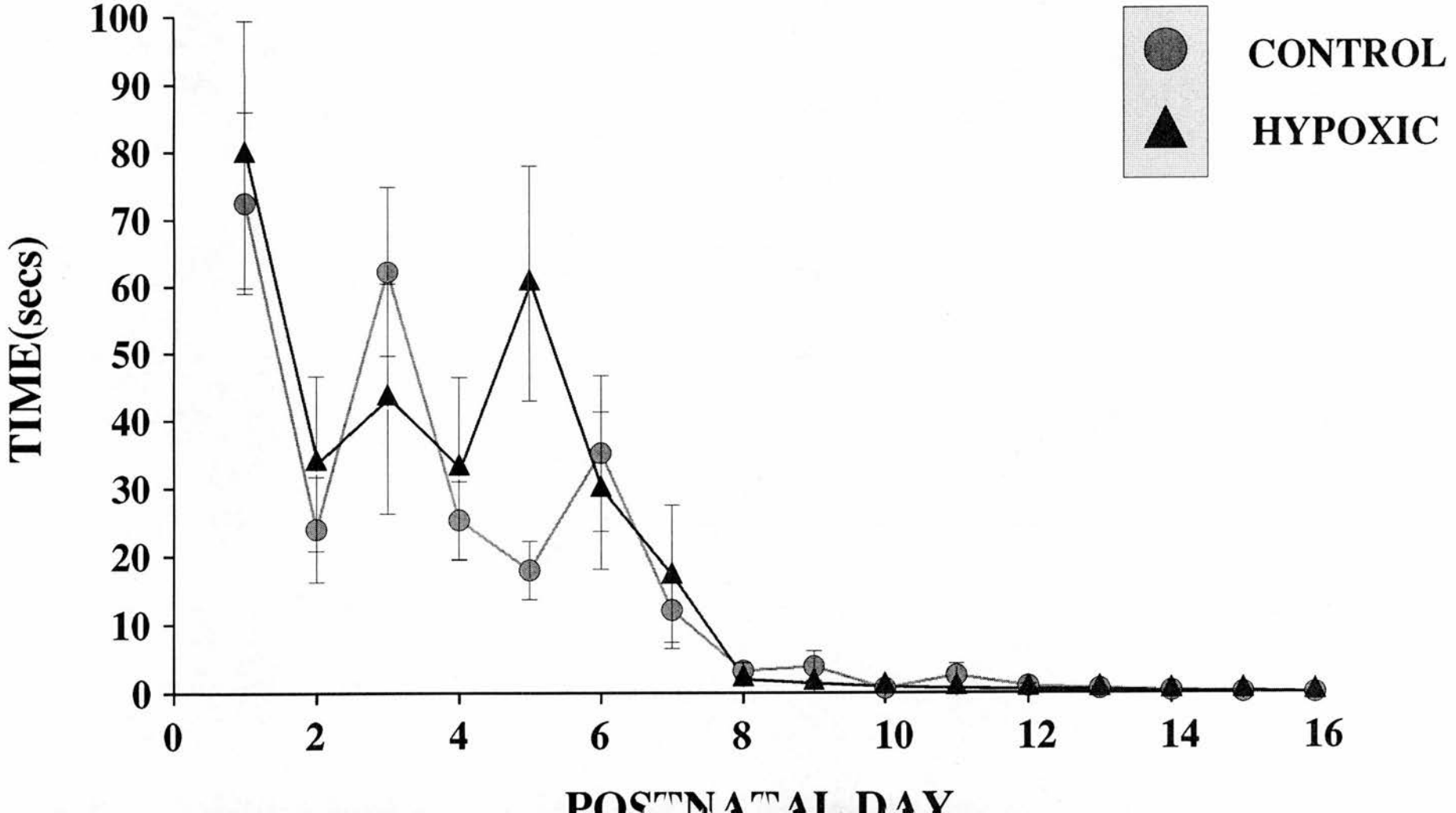
The graphs illustrate the daily measurements of the time to achieve a corrected body position from supine to upright. The neonatal rats were placed on their backs and given a maximum of 120 seconds to turn over. The measurements were made from pnd1 until pnd16 by which time all rats were able to right themselves. All measurements were made between 9.30 a.m. and 12.00 p.m.

The hypoxic rats had been exposed to a 30 minute occlusion, in utero, followed by a 20 minute recovery period and the control rats were the littermate controls for each hypoxic rats. 2 hours after delivery by Caesarian section the Lister-hooded pups were cross-fostered onto lactating Cob Wistar dams.

The two groups were compared statistically using the unpaired t-test but no significant differences were found between the control and the hypoxic rats.

FIGURE 3.12

RIGHTING REFLEX



3.3.5 Activity Measurements During the Postnatal Development of Rats Exposed to an *in utero* Hypoxic Insult

All of the following measurements are based on 3 minute observations made in an open-field environment.

a) The level of activity of neonatal rats from pnd1 to pnd11

A 4 point scoring system was used to record activity in the early postnatal period and the data are summarised in table 3.22. On pnd1 the majority of rats slept through the observation period and any movement was restricted to lifting the head and moving it from side to side. As described in section 3.3.4 crawling began on pnd5 and the increase in the activity score reflects this. By pnd11 there was a noticeable increase in activity with the rats frequently crawling round and round the edge of the observation area. No significant differences in activity were found between the control and the hypoxic rats.

b) The level of activity of neonatal rats from pnd11-pnd42

The 4 point scoring system was inadequate after pnd11 and activity was therefore measured according to the number of quadrants crossed in a 3 minute observation period. Table 3.23 summarises the data recorded for the control and the hypoxic rats.

From pnd11 to pnd15 there was a gradual increase in the number of quadrant crossings for both groups. The big increase on pnd17 reflects the improvement in co-ordination described in section 3.3.4. Activity in both groups peaked at pnd19 and then fell to a consistent level from pnd23 onwards. It is interesting to note a decrease in quadrants crossed on pnd22 which was the day after weaning. The lower number of quadrant crossings after pnd23 was because of the rats spent more of their time investigating the observation box and surrounding areas. For all measurements the majority of quadrant crosses were recorded in the first minute which reflects the exploratory behaviour of the pups when they were transferred from the habituation box to the adjacent observation box. After pnd28 there were very few if any quadrants crossed after the first minute of observation.

TABLE 3.22 THE LEVEL OF ACTIVITY IN NEONATAL RATS FROM PND1 TO PND11

PND	ACTIVITY SCORE (MEAN)	
	CONTROL RATS (n = 14)	HYPOXIC RATS (n = 8)
1	0.7 ± 0.2	0.6 ± 0.2
3	1.3 ± 0.3	1.8 ± 0.4
5	2.1 ± 0.3	2.0 ± 0.3
7	2.4 ± 0.2	2.4 ± 0.2
9	2.5 ± 0.2	2.8 ± 0.2
11	2.6 ± 0.1	2.6 ± 0.3

The table shows a summary of the mean ± s.e.m. activity ratings for control and hypoxic rats from pnd1-pnd11.
The scoring system was based on three minute observations of each pup in isolation. Any activity was then awarded a score according to a 4-point scale where:-

0 = No movement, except breathing, for > 1.5 mins.
1 = Very limited movement for > 1.5 mins with no change of location.
2 = Limited crawling or walking for < 1.5 mins.
3 = A lot of movement, crawling or walking for > 1.5 mins.

The data were subjected to unpaired t-tests but no significant differences were detected.

TABLE 3.23 THE LEVEL OF ACTIVITY, DURING 3 MINUTE OBSERVATIONS, OF NEONATAL RATS BETWEEN PND 11 PND 42

DAY	MEAN NUMBER OF QUADRANT CROSSES	
	CONTROL RATS	HYPOXIC RATS
11	3 ± 1	2 ± 1
13	3 ± 1	3 ± 1
15	10 ± 1	14 ± 2
17	29 ± 3	30 ± 1
19	35 ± 3	42 ± 4
21	29 ± 2	28 ± 3
22	25 ± 2	21 ± 3
23	29 ± 2	23 ± 3
25	27 ± 1	28 ± 4
28	27 ± 2	28 ± 2
33	26 ± 1	26 ± 2
36	29 ± 1	28 ± 4
39	29 ± 2	27 ± 4
42	28 ± 1	27 ± 2

The table shows the mean (± s.e.m.) number of quadrants crossed during a 3 minute period for control and hypoxic rats.

The data were subjected to unpaired t-tests but no significant differences were found between the control and hypoxic group of rats.

3.3.6 Functional Assessment of Adult Rats that were Exposed to a Pre-natal Hypoxic Insult

An assessment of the spatial memory in adult rats was made using a place-navigation task. Three groups of rats were trained to find a hidden platform in an open field water-maze; the groups were control rats (n = 14), hypoxic rats (n = 8) and hippocampal lesion rats (n = 4).

a) Trial latencies

The escape latencies for each of the 24 trials are illustrated in figure 3.13. The mean (± s.e.m.) escape latency for each of the 4 days of training for each group of rats is shown in table 3.24.

TABLE 3.24	MEAN ESCAPE LATENCY (secs)		
	GROUP		
DAY OF TESTING	CONTROL	HYPOXIC	HIPPOCAMPAL LESION
1	50.0 ± 4.6	51.7 ± 6.2	77.6 ± 8.6
2	21.7 ± 2.9	23.7 ± 4.8	45.5 ± 8.4
3	10.6 ± 1.6	14.3 ± 2.7	35.8 ± 7.2
4	8.0 ± 0.8	11.9 ± 2.7	16.2 ± 2.8

During the first trials the rats swam aimlessly around the pool and if the platform was found it was by chance rather than design. The rats spent the inter-trial interval sitting on their haunches and looking around the testing room. By the sixth trial there was a dramatic improvement in the performance of both the control and the hypoxic rats such that they swam in the direction of the platform as soon as they were released. The hippocampal lesioned rats swam in a distinctive circular pattern round and round the pool frequently missing the platform. After six trials the hippocampal lesioned rats were still swimming in circles and taking a long time to find the platform. By the end of the second day of training (trials 7-12) the majority of the control and the hypoxic rats found the platform in less than 20 seconds. The lesioned rats had improved a little but still took longer than the other two groups of rats. On the final day of training (trials 19-24) all 3 groups of rats were able to find the platform in less than 20 seconds.

ANOVA of all the data showed that the acquisition of the task was achieved by all 3 groups of rats but the lesioned rats took a significantly longer time to learn the exact location of the platform ($F(2, 23) = 6.53$; $p = 0.005$). There was also a significant effect of trials ($F(23, 529) = 17.47$, $p < 0.001$) but no significant group x trial interaction ($F(46, 529) = 1.12$, $p = 0.27$). Post-hoc comparisons, following ANOVA, showed that the lesioned rats took significantly longer to acquire the task than both the control and the hypoxic rats. In contrast, there was no significant difference between the control and the hypoxic rats.

b) Transfer test

Following acquisition of the task the rats were given a transfer test where the platform was removed from the pool. The performance level in this task was measured by the percentage of the 60 second testing time spent in each quadrant. The data from the transfer tests are summarized in figure 3.14. The control and the hypoxic rats had retained knowledge of the platform position and consequently spent the majority of the testing period (control = 57% and hypoxic = 51%) searching for the escape platform in the training quadrant ($p > 0.05$). As figure 3.15 shows, the swim paths of a control and a hypoxic rat are centred around the known location of the platform. After 30 seconds the rats began to swim outside the training quadrant presumably looking for an alternative platform location. The swim speeds of the control rats ($0.24ms^{-1}$) and hypoxic rats ($0.26ms^{-1}$) during the transfer test were similar ($p > 0.05$).

ANOVA showed a significant effect of the quadrant ($F(3,69) = 31.22$, $p < 0.001$) and a significant group x quadrant interaction ($F(6,69) = 4.80$, $p < 0.001$). Exploration of the simple main effect showed a significant effect of performance in the training quadrant ($F(2,70) = 12.16$, $p < 0.001$). Post-hoc comparisons found no significant difference between the performance of the control rats and the hypoxic rats during the transfer test but the rats with hippocampal lesions spent significantly less time in the training quadrant than the control and the hypoxic rats. Figure 3.15 shows that the hippocampally lesioned rats swam mostly in circles around the pool and overall the lesioned rats spent only 31% of the testing time in the training quadrant.

c) Histology

Photomicrograph 3.19 illustrates the extent of the hippocampal lesion created by multiple injections of ibotenic acid. The neurons of the CA3, CA2 and CA1 regions were all destroyed by the excitotoxin. Dentate gyrus granule cells and the subiculum were largely undamaged by the lesion.

3.3.7 Discussion

The experiments described in this section have demonstrated that rats exposed to a 30 minute *in utero* hypoxic insult suffer no long-term developmental or behavioural deficits. However, the exposure to an *in utero* hypoxic insult did significantly reduce a foetal rat's chance of survival after delivery by Caesarian section (see Table 3.20). The cerebral metabolic disruption associated with the hypoxic insult (see Section 3.1) may have been accompanied by extensive metabolic disturbances elsewhere in the foetal rat, e.g. the heart, thereby restricting the animal's capacity for recovery. This observation is supported by the fact that the heart was no longer beating in those animals which could not be resuscitated and is consistent with previous studies (Dawes *et al*, 1959; Myers, 1977). In addition, the effects of the 30 minute occlusion persisted for at least 24 hours because fewer hypoxic than control rats survived the cross-fostering procedure.

In the immediate post-natal period the influence of hypoxia was evident not only in the high mortality rates but also in the lethargy and, to some extent, the convulsive activity of the hypoxic rats. The fact that no differences, between the control and hypoxic rats, were seen 2 hours after delivery coincides with the recovery of brain energy metabolites discussed in section 3.1.

The physical development of the hypoxic rats from pnd0 to adult life could not be distinguished from that of their littermate controls. Previous studies have shown that perinatal asphyxia or anoxia can be associated with restricted weight gain particularly in the first 3 weeks of post-natal life (Windle and Becker, 1943; Bjelke *et al*, 1991; Vannucci and Duffy, 1976). This apparent conflict with previous studies is

probably due to methodological differences in the induction of perinatal asphyxia. Although cerebral metabolic alterations reported by Vannucci and Duffy (1976) following induction of anoxia in a 100% N_2 atmosphere are of a similar magnitude to those found in this study the weight discrepancies observed were only reported at pnd30 and may not have persisted into adult life.

The rate at which physical development proceeded in both control and hypoxic neonatal rats is in accordance with existing studies of normal laboratory rat development (Bolles and Woods, 1964; Altman and Sudarshan, 1975). Any differences between this and the previous studies were minimal and were largely absent after pnd10 and so the maturation of the righting reflex, the onset of quadruped walking and the improvement in rearing and balance all occurred at the expected times. The absence of significant differences between this and previous studies of development suggests no long-term deficit associated with the prenatal surgical procedure (and exposure to anaesthetic), with the hypoxic insult or with the cross-fostering procedure.

Activity measurements from pnd1 to pnd42 found no significant differences between the control and the hypoxic rats. In support of this observation Bjelke and Colleagues (1991) recently reported that prenatal asphyxia, created by submersion of the rat uterus, had no effect on locomotor activity. Also repeated prenatal exposure to hypoxia had no significant effect on locomotor activity in the adult (McCulloch and Blackman, 1976).

In contrast, several studies have shown that exposure to anoxia in the early postnatal period causes a transient phase of hyperactivity between pnd20-42 (Hershkowitz et al, 1983; Dell'Anna et al, 1991; Nyakas et al, 1991). Rearing and sniffing activity rather than locomotion are the main features of hyperactivity, and this contrasts with a study in which prenatal asphyxia was shown to decrease rearing activity (Bjelke et al, 1991). Rapid development of neurotransmitter systems and interconnections during the late prenatal and early postnatal periods are likely to govern the range of motor responses to environmental manipulation. The suppression or enhancement of activity is entirely dependent upon the age at which the animal was

exposed to hypoxia/anoxia such that the pattern of vulnerability of different transmitter systems at different critical stages of development reflects the outcome of the hypoxia/anoxia. Evidence for the involvement of dopaminergic, cholinergic and noradrenergic neurotransmission in these altered motor responses has been provided from exposure to pre- and post-natal hypoxia/anoxia (Hershkowitz et al, 1983; Speiser et al, 1988; Bjelke et al, 1991). Individual transmitter systems were not investigated in this study but a gross histological survey found limited evidence of neuroanatomical disruption which might account for the lack of effect of the hypoxia on postnatal activity.

The hippocampus is a region which would appear to be particularly vulnerable to hypoxia/anoxia in both the neonate and the adult and this has a significant influence on the performance of animal in tests of cognitive ability and spatial learning (Bjelke et al, 1991; Dell'Anna et al, 1991' Hershkowitz et al, 1983; Squire, 1992). Of particular interest for the investigation of models of human perinatal hypoxia is the durability of the impairment into adult life. When assessed in adult rats repeated exposure to prenatal hypoxia was found to significantly impair performance in a Hebb-Williams maze task (McCulloch and Blackman, 1976). Post-natal anoxia consistently results in poor performance in discrimination learning (Hershkowitz et al, 1983), in spatial memory tasks (Nyakas et al, 1991; Dell'Anna et al, 1991) and in passive and active avoidance learning (Speiser et al, 1988) and all of these impairments are long-lasting.

In this study rats which received a bilateral lesion of the hippocampal region were impaired in a place navigation task which is consistent with previous results (Morris et al, 1982; Morris et al, 1990). However, adult rats which had been exposed to the prenatal hypoxic insult were not impaired in either the acquisition or the retention of the place navigation task. The small reduction in adult hypoxic rat brain CA1 width demonstrated in section 3.2 does not appear to have caused any significant effect on the rat's performance in this task. This might suggest that the rat can withstand a minor alteration in the hippocampal formation without any functional impairment in spatial learning.

FIGURE 3.13 THE PERFORMANCE OF CONTROL, HYPOXIC AND HIPPOCAMPALLY-LESIONED RATS IN A WATER-MAZE - ESCAPE LATENCIES

The figure illustrates the mean (± s.e.m.) time taken to find a hidden platform (escape latency) in a 2m diameter water maze over 24 trials. Each rat was given 6 trials a day with a maximum time of 120 seconds per trial and an inter-trial interval of 30 seconds. The platform was located in the centre of either the NE or the SW quadrant and the rats were divided randomly to assign equal numbers to each platform position.

A tracking device followed the rat's position in the pool and the video signal was relayed to a video recorder and then to an image analyser (HVS VP112). The x and y co-ordinates of the rat's position were sampled at a frequency of 10Hz by an Archimedes computer using the 'Water-Maze' (c. R.G.M. Morris & R. Spooner) programme.

All the escape latencies were subjected to ANOVA which detected a significant difference between the control and the hippocampal rats and between the hypoxic and the hippocampal rats ($p < 0.05$), but not between the control and hypoxic rats.

FIGURE 3.13

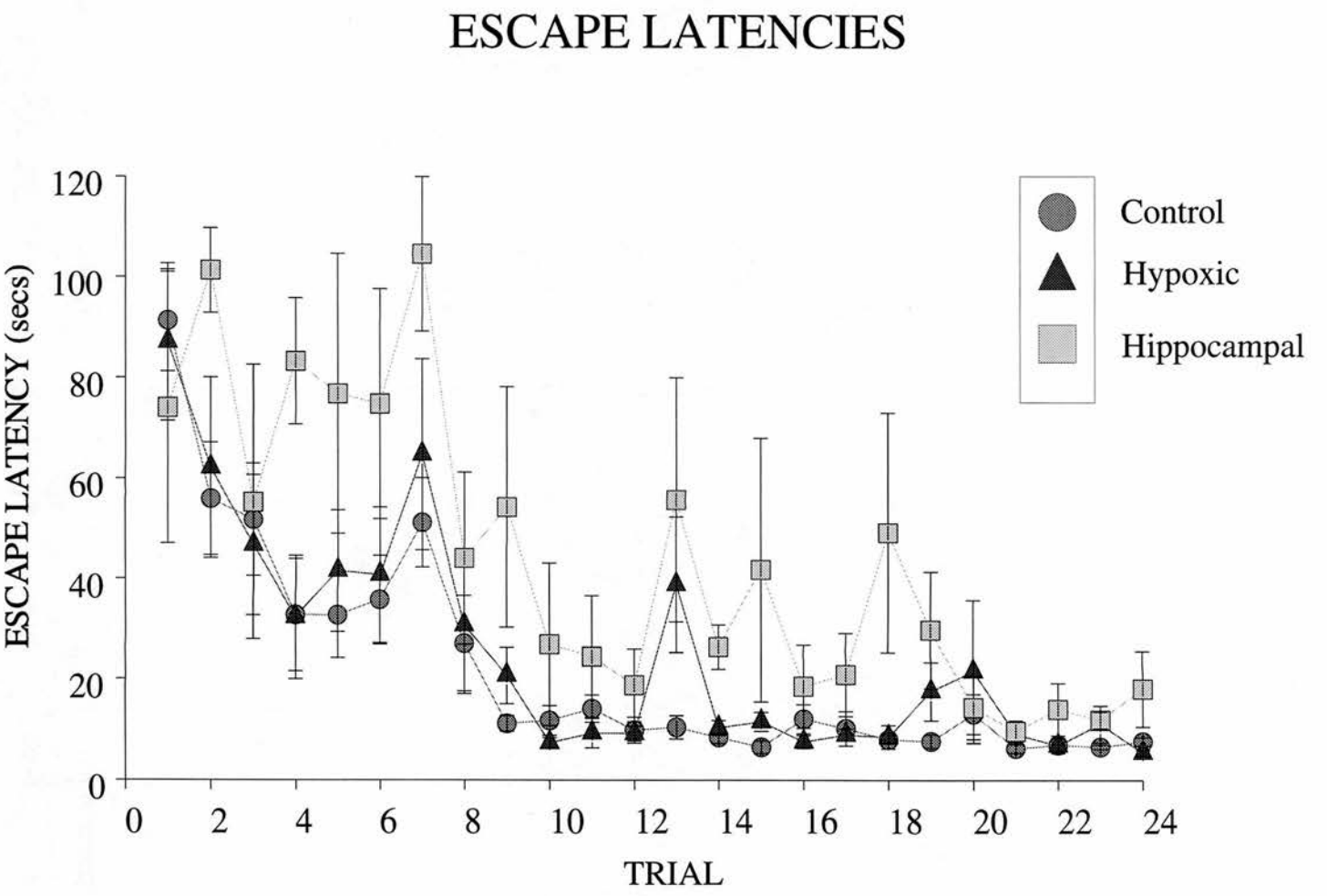
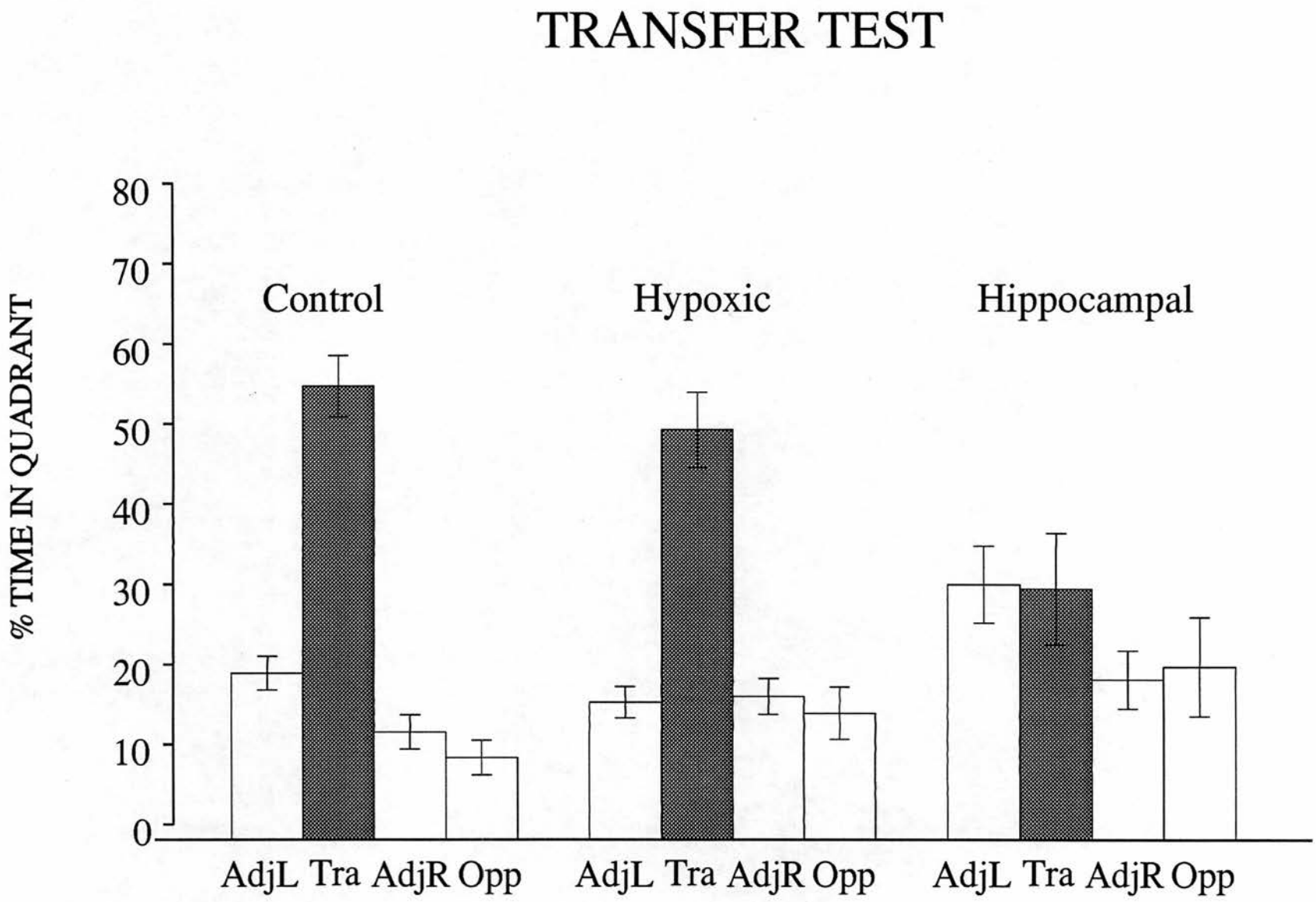

ESCAPE LATENCIES
ESCAPE LATENCY (secs)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TRIAL
Control
Hypoxic
Hippocampal

FIGURE 3.14 PERFORMANCE IN A TRANSFER TEST

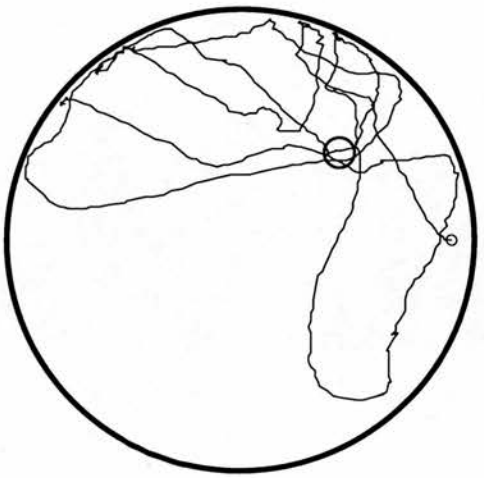
The histograms represent the proportion of a 60 second testing period each group of rats spent in each quadrant. For the transfer test the platform was removed and the rats were allowed to swim freely. The quadrant in which the platform was normally located was labelled the training quadrant (Tra).

The recording procedure is described in figure 3.13. All of the data were subjected to ANOVA which exposed a significant difference in the proportion of time spent in the training quadrant between the hippocampal and the control rats and between the hippocampal and the hypoxic rats ($P < 0.001$).

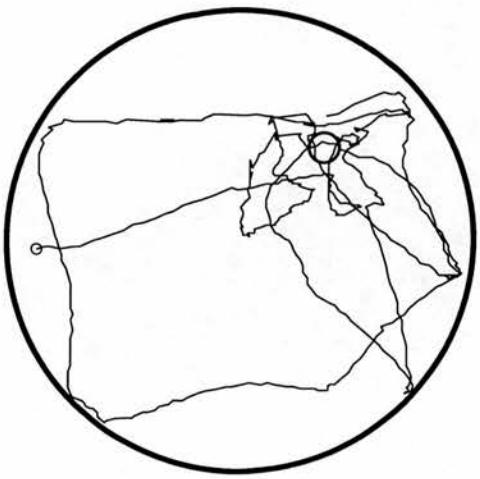
FIGURE 3.14


TRANSFER TEST
% TIME IN QUADRANT
80
70
60
50
40
30
20
10
0
Control
Hypoxic
Hippocampal
AdjL Tra AdjR Opp
AdjL Tra AdjR Opp
AdjL Tra AdjR Opp

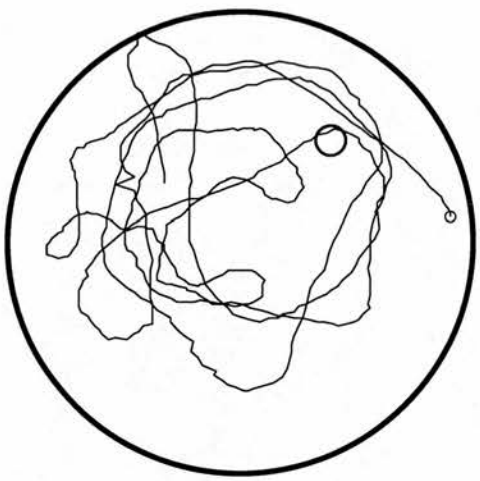
GURE 3.15 THE SWIM PATH OF A CONTROL, A HYPOXIC AND A RAT WITH A BILATERAL HIPPOCAMPAL LESION DURING THE TRANSFER TEST.



a) CONTROL RAT



b) HYPOXIC RAT



c) HIPPOCAMPAL-LESION RAT

The three figures show the path taken by three rats during a 60 second transfer test in which the platform had been removed from the pool. All three rats had been trained to locate the platform in the north east quadrant. A computer program called Watermaze sampled the x and y co-ordinates of the rats position with a frequency of 10Hz and then used the co-ordinates to produce the paths shown above. ANOVA showed the performance of the control and the hypoxic rats to be significantly better than that of the rats with bilateral hippocampal lesions ($p<0.001$).

3.19

Photomicrograph 3.19
The photograph illustrates the extent of the excitotoxic ibotenic acid lesion in the hippocampal regi
The lesioned rat was perfusion fixed using 10% buffered formalin and 30µm frozen brain sections w
stained using cresyl fast violet. Magnification = 12x.

CHAPTER FOUR

GENERAL DISCUSSION

4. GENERAL DISCUSSION

4.1 A NOVEL MODEL OF PERINATAL HYPOXIA

A reliable model of *in utero* hypoxia in the near-term foetal rat has been developed after a great deal of manipulation of the experimental variables. The term hypoxia must, however, be applied with some caution. In this model the occlusion of the uteroplacental vessels left the foetal circulation intact, but restricted the supply of maternal arterial oxygen to the foetuses resulting primarily in a hypoxic trauma. However, prolonged myocardial hypoxia in the foetus might also jeopardise cardiac function and result in systemic hypotension (Myers, 1977; Guyton, 1991). The addition of reduced cerebral perfusion pressure to the initial hypoxic insult in all probability extends the cerebral metabolic dysfunction observed in the foetal rat brains (Duffy *et al*, 1972; Siesjö, 1978, 1984). The possibility that the insult was a combination of hypoxia and ischaemia to both the brain and myocardium may explain the high mortality rates, and inspection of rats that could not be resuscitated showed the heart to be motionless. Furthermore, the 75-80% reductions in foetal rat brain ATP and PCr levels noted in the present study far exceed changes in high energy phosphates induced by hypoxia alone in adult rat brain (Duffy *et al*, 1972), and this may be indicative of an additional ischaemic or hypotensive insult.

The possible existence of hypoxic-hypotension in the anaesthetised, supine mother (without the aid of a respirator) might be another factor for consideration, particularly with respect to the observed cerebral metabolic changes in both the sham-operated and the control foetal rat brains. In order to restrict the duration of anaesthesia to the absolute minimum it was decided not to take regular samples for blood gas analysis of the maternal circulation, but previous studies have reported a lowering of systemic blood pressure during inhalation anaesthesia particularly when the rat is in the supine position (Brann and Myers, 1975; Myers, 1977). Since anaesthesia was as short and light as possible, cumulative insults in the hypoxic animals where maternal arterial supply was restricted for the majority of the procedure were unlikely. In future experiments it may be worthwhile to consider a series of investigations into the blood

gas and blood pressure changes both in the foetus and the dam so that some of these issues can be resolved.

The model of *in utero* hypoxia described in the thesis is associated with considerable cerebral metabolic disruption; the decline in ATP and PCr suggest a severe energy debt, and the fall in glucose with accumulation of lactate suggest anaerobic glycolysis predominates without oxidative phosphorylation. The high numbers of hypoxic rats that cannot be resuscitated, particularly after a 30 minute occlusion, demonstrates the severity of the insult. Yet within three hours of delivery by Caesarian section brain energy metabolites in the surviving animals have recovered to control levels, and as recovery proceeds the neonatal hypoxic rats become less and less distinguishable from their litter-mate controls. Thus, the surviving animals appear to suffer no gross neuropathological lesion, their physiological development and reflexes are normal, and there are no apparent behavioural abnormalities related to motor or cognitive deficits.

4.2 RESISTANCE OF THE PERINATAL RAT BRAIN TO HYPOXIC INSULTS

These results beg the question of how the near-term foetal rat can withstand such a severe cerebral metabolic insult with no apparent ill-consequence. To answer this question it is pertinent to discuss putative mechanisms of resistance to the trauma and to contrast these with the purported mechanisms associated with hypoxic-ischaemic neuronal necrosis.

The ability of foetal and neonatal animals to survive much longer in an anoxic environment than their adult counterparts has been demonstrated repeatedly (Fazekas *et al*, 1941; Duffy *et al*, 1975). The mechanisms which aid their resistance to oxygen deprivation include lower cerebral energy requirements and lower rates of glucose uptake and metabolism in the foetal brain compared with adult animals (Duffy *et al*, 1975; Vannucci *et al*, 1989; Kjellmer, 1991). The accumulation of brain tissue lactate above 20nmols/mg brain tissue following a 30 minute occlusion period is equivalent to changes observed in adult animal models of transient global ischaemia, and is greater

than changes found in the 7-day old rat model of unilateral hypoxic-ischaemia (Rehncrona *et al*, 1981; Siesjo, 1984; Allen *et al*, 1988; Palmer *et al*, 1990). However, in the case of the foetal rat brain the ability to utilise lactate as well as glucose as a metabolic substrate and to selectively increase the uptake of lactate from blood to brain during recovery may prevent the serious pathological effects of lactate accumulation associated with adult hypoxia (Moore *et al*, 1971; Thurston and McDougal, 1969; Vannucci *et al*, 1989; Cremer, 1982; Palmer *et al*, 1990). The preferential utilisation of lactate for oxidative phosphorylation explains the rapid recovery of brain tissue lactate and the increase in brain tissue glucose during early postnatal recovery.

The foetal rat exists in a state of hypercapnia and the depressant action of raised carbon dioxide tension is known to favourably reduce metabolic rates and restrict the decline in energy stores (Vannucci and Duffy, 1976). During the hypoxic insult the inability to maintain ATP supplies results in failure of cells to undertake metabolic work (Siesjö, 1984), but since metabolic requirements are lower than the adult and are no doubt suppressed by raised carbon dioxide levels, this may not be an important feature of the insult. However, at this critical stage of development when rates of protein synthesis and lipid deposition are high, and when several regions of the rat brain have yet to achieve their final pattern of organisation (e.g. cerebellar cortex and hippocampus, Altman, 1972; Bayer, 1980), a prolonged reduction in brain ATP such as in the 30 minute occlusion may cause irreversible damage although not necessarily neuronal necrosis (Hicks *et al*, 1962).

The extent and severity of the metabolic deficit in the *in utero* model must also be compared with neonatal and adult models of hypoxic-ischaemia. In adult animal models a significant reduction in CBF below 12-15ml/100g/minute is associated with a total depletion of both PCr and ATP within the first 5-7 minutes of the insult (Siesjö, 1978, 1984; Rehncrona *et al*, 1981; Naritomi *et al*, 1988; Allen *et al*, 1988). If the ischaemic insult is reversed with complete restoration of perfusion pressure brain, PCr levels recover within 15 minutes but ATP concentrations can remain below control

levels for a considerable period of time (Siesjö, 1984). Revival time, or the maximum survivable period of ischaemia, is critically linked to the extent of the ATP depletion during the insult and the failure to recover basal ATP levels in the early reflow phase (Siesjö, 1984). The maximal 84% depletion in brain tissue PCr and 78% reduction in brain tissue ATP following a 30 minute occlusion noted in the present study are not as extensive as changes observed in adult ischaemia yet are similar to changes reported in the 7-day old rat model of hypoxic-ischaemia (Palmer *et al*, 1990). However, in the neonatal rat model ATP only recovers to 68-81% of control levels at 72 hours post-insult (Palmer *et al*, 1990). The complete restoration of brain tissue ATP within 60 minutes in the *in utero* hypoxia model described in this thesis contrasts with data from neonatal and adult models of hypoxic-ischaemia. The restoration of ATP in this model is an important feature that might be related to the lack of extensive neuronal damage.

The level of foetal rat brain tissue PCr is approximately 45 times lower than that in the neonate, and low levels of energy reserves in the foetal rat may reflect a low level of electrical activity (Himwich, 1962; McIlwain and Bachelard, 1985). K^+ ion homeostasis can be identified in the neonatal rat CSF at birth but considerable variations in brain K^+ and Na^+ ion concentration occur during the first week of postnatal life in concert with a significant rise in ATPase activity (Jones and Keep, 1982; Himwich, 1962; Jones and Rolph, 1985; McIlwain and Bachelard, 1985). These changes in ion homeostasis and ATPase activity are coincident with the development of the rat EEG between pnd 10-20, with dendrite formation and establishment of synaptic contacts, and with increases in both neurotransmitters and enzymes associated with transmitter metabolism (Himwich, 1962; McIlwain and Bachelard, 1985; Aghajanian and Bloom, 1967; Jones and Rolph, 1985). The consequence of failure to provide ATP for ATPase-governed membrane ion pumps is presumably much more severe in the 7-day old neonatal and adult rat brain compared with the foetal rat brain as a result of these postnatal alterations.

The excitotoxic property of glutamate, in combination with elevated extracellular glutamate concentrations during ischaemia, is thought to be one of the

destructive factors in hypoxic-ischaemic cell death (Rothman and Olney, 1986; Hagberg *et al*, 1985; Choi, 1990). Although extracellular glutamate levels have been shown to rise during occlusion of the umbilical cord in foetal sheep (Hagberg *et al*, 1987), glutamatergic innervation must be intact for hypoxic/ischaemic cell death to occur in adult animals (Benveniste *et al*, 1989). The full pattern of glutamatergic innervation only develops during the first postnatal week (Campuchiaro and Coyle, 1978), and extensive studies have demonstrated that both the development of the characteristic actions of adult glutaminergic synaptic transmission within the hippocampus, and the formation of the complex intrahippocampal pathways, occur postnatally in the rat (Baudry *et al*, 1981; Amaral and Dent, 1981; Bayer, 1980). Moreover, very low densities of excitatory amino acid receptors have been detected at birth (Insel *et al*, 1990; Miller *et al*, 1990), and the prodigious rise in the density of the NMDA and AMPA subtypes of excitatory amino acid receptor during early postnatal life correlates with a pronounced sensitivity to relevant excitotoxins that far exceeds that seen in the adult (Insel *et al*, 1990; Tremblay *et al*, 1988; McDonald and Johnston, 1990). Thus, a majority of the features of glutamatergic neurotransmission required for expression of the excitotoxic action of glutamate in adult rat models of hypoxic-ischaemia develop during the postnatal period. In this perinatal rat model of hypoxia the proven integrity of brain regions sensitive to the excitotoxic action of glutamate in adult animals (e.g. CA1 pyramidal neurons) may therefore be due to the developmental immaturity of glutamatergic neurotransmission. However, another model of perinatal asphyxia in the rat has reported CA1 cell loss and methodological considerations cannot therefore be ruled out (Bjelke *et al*, 1991).

GABA is the main inhibitory neurotransmitter in the adult CNS. However, during development GABA has been shown to elicit excitatory responses in the CA3 region of the rat hippocampus (Ben-Ari *et al*, 1989). These excitatory responses take the form of giant depolarising potentials and are believed to be mediated by reversed Cl^- movements made possible by reversed operation of the Cl^- membrane pump (Cherubini *et al*, 1991). Immunoreactivity to the GABA producing enzyme glutamic

acid decarboxylase has been demonstrated in the prenatal period (Amaral and Kurz, 1985), and the sensitivity to exogenous GABA application noted in electrophysiological experiments is present on the day of birth (Ben-Ari *et al*, 1989). The functional significance of GABAergic transmission at this developmental stage is believed to be related to differentiation and synaptogenesis of hippocampal neurons (Cherubini *et al*, 1991). Excitation evoked by GABA may increase the intracellular concentration of Ca^{2+} (Yuste and Katz, 1991), and this is known to be essential for the regulation of growth and development of neurons and their processes (Nitecka *et al*, 1984). Excessive influx of both Cl^- and Ca^{2+} ions into hippocampal CA3 neurons may therefore occur should the metabolic disruption caused by the *in utero* hypoxic insult increase extracellular GABA concentrations. The long-term consequences of severe disruption of Ca^{2+} homeostasis are known to result in cellular necrosis (Siesjö and Bengtsson, 1989; Choi, 1990), and this may be related to the higher number of darkly stained cells in the hypoxic CA3 region noted in the present study. In support of this theory, pretreatment with the Ca^{2+}-channel blocker nimodipine in a model of postnatal anoxia reduced deficits in spatial learning normally associated with damage to the hippocampal region (Nyakas *et al*, 1991).

It would appear that the foetal rat brain is able to offer some level of resistance against many of the metabolic and excitotoxic mechanisms thought to cause hypoxic-ischaemic cell death in the 7-day old neonate and adult rat brain. In addition, several other factors of cerebral resistance should be considered. The postulated neuroprotective effect of NGF (Shigeno *et al*, 1991), a neurotrophic agent for which receptors have been identified in the developing hippocampus (Lu *et al*, 1989), may provide additional resistance on E22 during a phase of considerable hippocampal growth (Bayer, 1980). Of relevance to this idea is the observation that NGF levels are raised following exposure to hypoxia in the adult rat brain (Lorez *et al*, 1989). The postulated neuroprotective effect of hypothermia (Busto *et al*, 1989a; Yager *et al*, 1993) is in all probability likely to have the most significant effect in this paradigm where some element of heat loss could not be avoided. A neuroprotective effect of mild

hypothermia has been demonstrated in adult models of global and focal ischaemia even if the hypothermia is induced in the immediate recovery period (Busto *et al*, 1987; Busto *et al*, 1989a). In the 7-day old rat model of unilateral hypoxic-ischaemia when cerebral temperature was reduced to 34°c during the hypoxia only 30 % of the animals exhibited brain injury compared with 90 % when temperature was maintained at 37°c (Yager *et al*, 1993). A further reduction to 31°c afforded complete protection from hypoxic-ischaemic neuronal injury. However, when hypothermia was induced after the hypoxia no neuroprotective action was demonstrated. In the model described in this thesis foetal body core temperature was reduced to 34°c following 30 minutes of the *in utero* trauma. This hypothermia may be another reason for the lack of extensive brain injury. In support of this hypothesis, in the asphyxia model described by Bjelke and colleagues (1991) submersion of the entire uterus in a heated water bath would serve to maintain normothermia, and this may explain the disparity between the necrotic effects noted in that model compared with the lack of extensive neuropathological damage described here.

In summary, the resistance of the near-term foetal rat brain to an *in utero* hypoxic insult induced by uteroplacental vessel occlusion is likely to be related to several factors such as low energy requirements, the hypercapnic suppression of metabolic rates, the rapid and complete recovery of high energy compounds, and the immaturity of synapse formation and responses to neurotransmitters within the CNS. With all of these features in mind it is difficult to fully explain the paradox of the high mortality rates compared with the preservation of brain function and development in surviving animals. However, one must presume that the foetal resistance to hypoxic insults has a limit perhaps governed by secondary ischaemia and metabolic disruption of the respiratory centres located in the hind brain. Although the rapid recovery of brain energy metabolites is primarily governed by the ability of foetal brain to utilise both lactate and glucose as metabolic substrates, there must remain some element of compromise to the foetal rat because a significantly greater number of hypoxic rats died during the first 24 hours of neonatal life. Exhaustion of cardiac (and brain) stores of

glycogen during the hypoxic insult (Dawes *et al*, 1959; Vannucci and Duffy, 1974) may jeopardise efficient cardiac function resulting in secondary insults that mitigate against short-term survival of the neonatal rats.

4.3 EVALUATION OF THE MODEL

In this paradigm hypoxia does not result in significant long-term neuropathological or physiological changes in surviving animals raising the question of whether the use of this procedure to model human perinatal brain injury can be justified. With respect to the available alternatives the answer must be no. The induction of a unilateral infarct in the 7-day old rat model of hypoxic-ischaemia (Rice *et al*, 1981) is useful because at this age the development of the rat brain is comparable with a human neonate at birth, and also because this model is useful as a tool for screening potential neuroprotective agents (see Vannucci, 1990a). However, the gross unilateral lesion created in the 7-day old rat is far more extensive than the post-mortem hypoxic-ischaemic brain damage seen in the human infant (Hill and Volpe, 1981; Larroche, 1985). Only a small proportion of those children exposed to a hypoxic-ischaemic insult in the perinatal period suffer serious physical and/or mental handicap (Levene, 1987; Hull and Dodd, 1992). It has been suggested that hypoxic-ischaemia, or perinatal asphyxia, in isolation is rarely the direct cause of disorders such as cerebral palsy (Scott, 1976; Thomson *et al*, 1977; Blair and Sanley, 1988; Naeye *et al*, 1989). The high level of foetal brain tolerance to hypoxia demonstrated in this thesis and previous animal models (Fazekas *et al*, 1941; Stafford and Weatherall, 1960) may also be found in the human foetal brain, and probably accounts for the excellent prognosis of those compromised infants who are successfully resuscitated within the first 10-15 minutes after birth (Levene, 1987; Kjellmer, 1988). The occurence of birth asphyxia severe enough to cause brain damage generally has two possible outcomes; death or a lack of significant neurological injury (Naeye *et al*, 1989).

The model described here along with existing models of total and partial asphyxia in foetal animals are of genuine interest for reproducing the environmental

conditions of prenatal and birth injury. Of particular relevance is the frequent occurence of arrest in uterine blood flow during the second stage of labour (Jensen, 1992). However, the wide range of effects reported in these studies demonstrates the difficulty in translating experimental investigations of foetal animals to the diagnostic and therapeutic applications of the clinic. The main contrast is the lack of neuropathological damage to the periventricular white matter, cerebellar Purkinje cells, cortical layers 3 and 5, the inferior colliculus and other regions known to be sensitive to hypoxic-ischaemia in the human infant (Hill and Volpe, 1981; Larroche, 1985; Levene, 1988). The small but significant reduction in hippocampal CA1 width in hypoxic rat brains might be taken to indicate some long-lasting influence of the perinatal trauma and as such is consistent with the well known vulnerability of the hippocampus to hypoxic-ischaemic injury. The neovascularisation demonstrated in the hind brain is one feature that does correspond to pathological events found in the post-mortem human infant brain following perinatal hypoxic-ischaemia (Personal Communication, J.Bell, Department of Neuropathology, Western General Hospital, Edinburgh, U.K.).

The cerebral metabolic disruption demonstrated in this thesis is extensive but short-lived which might imply that imaging of high energy phosphate compounds in the asphyxiated neonate using magnetic resonance image or PET scanning need not necessarily reflect the true extent of the insult. In addition, measurements of plasma and CSF lactate may be of little benefit in the detection of perinatal hypoxic-ischaemic brain damage because lactate can be used as a metabolic substrate in foetal brain. Such measurements may, however, provide a useful indication of the duration and severity of the hypoxic-ischaemic episode. The analysis of mortality figures shows that the duration of the hypoxic insult is a critical determinant of survival in the neonatal rat. Thus, in the clinic a method or device that determines the length and severity of the trauma will be of more use than methods that simply diagnose the presence of an hypoxic-ischaemic insult.

It has been shown that the surviving rats endure a hypoxic insult with no

apparent ill-consequence and concurrently a large proportion of those human infants who are exposed to perinatal hypoxic-ischaemia are able to grow into healthy intact adults (Levene, 1987; Hull and Dodds, 1992). In those cases where the child exhibits the severe, grade III encephalopathy the use of real-time ultrasound and PET scans can accurately detect extensive cerebral disruption which is frequently accompanied by haemorrhage (Volpe and Pastemak, 1977; Volpe *et al*, 1985; Levene, 1987). The model presented here, and in particular the lack of haemorrhagic lesions, might suggest that a single hypoxic insult is not sufficient to cause extensive brain damage. Children exhibiting severe brain damage with accompanying haemorrhage may therefore be exposed to additional risk factors together with hypoxic-ischaemia during gestation *in utero* or during passage through the birth canal.

A large number of rats died within 24 hours of the initial hypoxic insult, and this emphasises the clinical importance of continuous monitoring of physiological variables during the first few hours after exposure to the trauma (Volpe, 1977; Kjellmer, 1988). Since cardiovascular function appears to be a limiting factor, the need to assess foetal breathing movements and neonatal heart and lung function in both the diagnosis and treatment of hypoxic-ischaemia cannot be stressed enough (Stahlman, 1977; Levene, 1987). Thus, it is reassuring that the current emphasis of clinical research is on non-invasive methods of detecting brain injury such as magnetic resonance imaging, real-time ultrasound and PET scanning, and on areas such as foetal and neonatal cardiovascular function rather than biomedical measures of diagnosis (Volpe and Pastemak, 1977; Volpe *et al*, 1985; Kjellmer, 1988; Wyatt *et al*, 1989; Moore and Hanson, 1992).

Although the treatment of perinatal hypoxic-ischaemia must be multi-faceted to encompass possible injury to all body regions, there are presently few accepted modes of clinical care which protect the neonatal brain. Extensive investigations in adult and 7-day old neonatal rodents have demonstrated the potential therapeutic application of glutamate antagonists (Swan *et al*, 1988; Park *et al*, 1988; Bullock *et al*, 1990; Buchan *et al*, 1991; Nellgard and Wieloch, 1992; McDonald *et al*, 1987; Andine *et al*, 1988).

However, the immaturity of glutamatergic transmitter systems and the lack of glutamate receptors in the brains of perinatal animals suggests that the use of glutamate receptor antagonists may not be appropriate for the treatment of human perinatal hypoxic-ischaemia. GABA receptor antagonists might prove to be effective in reducing hippocampal injury if the excitatory action of GABA found in immature rat CA3 pyramidal neurons is also present in the neonatal human hippocampus. This has wide-reaching implications for the use of benzodiazepines in the treatment of neonatal seizures following hypoxic-ischaemia, and might explain the conflicting reports about the benefits of benzodiazepines (Vannucci, 1990a). Prevention of excessive Ca^{2+}-influx into vulnerable neurons using Ca^{2+} channel antagonists such as nimodipine or nifedipine may reduce the risk of hypoxic-ischaemic cell death, but the risk of manipulating intracellular Ca^{2+} levels at critical stages of neuronal development must be taken into account. Although temperature regulation and the use of hypothermia as a means of neuroprotection has always been controversial in the clinic (Miller, 1971; Levene, 1987), a lowering of body temperature should also be considered as one of several additive measures used to treat an asphyxiated infant.

Further manipulation of the model reported here might be more effective in producing neuronal necrosis and/or physical and behavioural effects. For example, one change might involve the occlusion of both uterine horns and then allowing the dam to give birth naturally. Although this would not produce littermate controls and would not allow monitoring of cerebral metabolic changes, these are factors well documented in this thesis. Alternatively, since multiple insults are known to be particularly damaging the use of three 10 minute occlusions might be effective in producing neuronal necrosis (Myers, 1977; Hill and Volpe, 1981; Levene, 1987).

The nature of this investigation was such that general surveys were made in three main areas. The subtle changes in hippocampal CA1 width suggest that the histological study could be extended to look at microglia responses to the insult, at transmitter systems (by immunocytochemistry and binding techniques), at axonal and dendritic process formation and at myelination in order to detect any further subtle

changes than were recognised in the current study. With respect to physical development, a closer examination of rearing behaviour and time spent sniffing is another extension which might be considered, particularly with respect to results reported in other models of perinatal hypoxia/anoxia (Bjelke *et al*, 1991; Dell'Anna *et al*, 1991; Nyakas *et al*, 1991). This investigation would be particularly relevant as a model of minimal brain dysfunction where hyperkinesis and learning disorders are not accompanied by extensive neuronal loss (Towbin, 1971; Wender, 1984). Further development of the model described in this thesis could therefore be of considerable use for the study of foetal tolerance, and thereby provide clues as to the optimal neonatal protection programme.

4.4 SUMMARY

This study describes the investigation of a model of *in utero* hypoxic-ischaemia in the near term foetal rat. The insult created a severe cerebral metabolic disruption that was not survived by a large proportion of rats. However, those rats that did survive displayed a high-level of resistance to the insult such that damage to the brain was discrete and not on the whole significant. The long-term surviving rats suffered no apparent physical, motor, behavioural or cognitive deficits.

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APPENDIX

APPENDIX RAT BRAIN HISTOLOGY

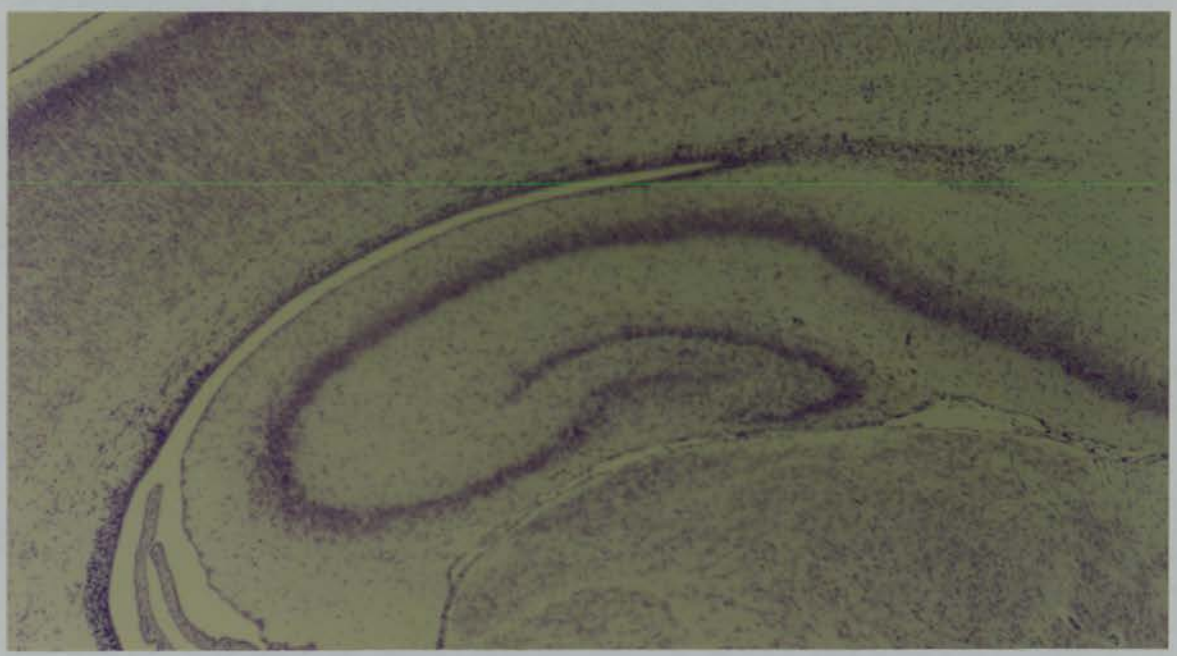
The following appendix contains additional examples of the hippocampal brain sections taken from control and hyopxic rat brains on pnd 3. The rats were perfusion fixed with FAM and then processed for wax-embedding. The brains were sectioned on a microtome at 6μm and then stained with cresyl violet.

Page 1. shows three control rat brains and page 2. shows three hypoxic rat hippocampi. The magnification of all the sections is 40x.

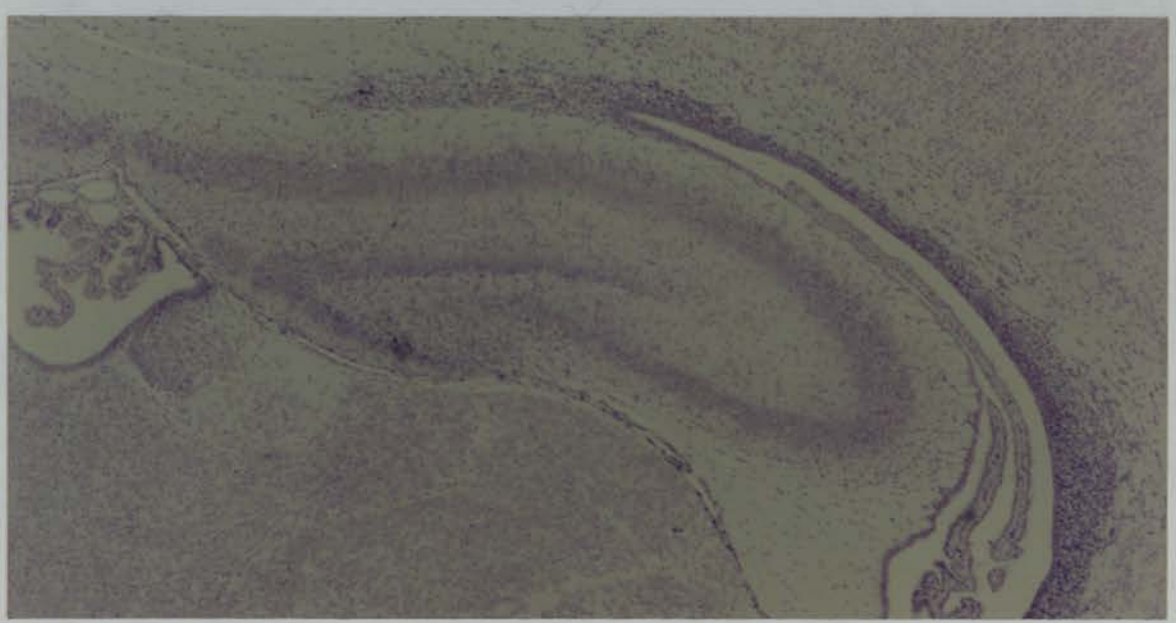
Page 3. shows the CA1 region of three control rat hippocampi and page 4. shows the CA1 region of three hypoxic hippocampi. The magnification of the sections is 200x.

Page 5. shows the hippocampal CA3 region of three control rat brains and page 6. shows three hypoxic CA3 sections. The magnification of the sections is 200x.

a



b



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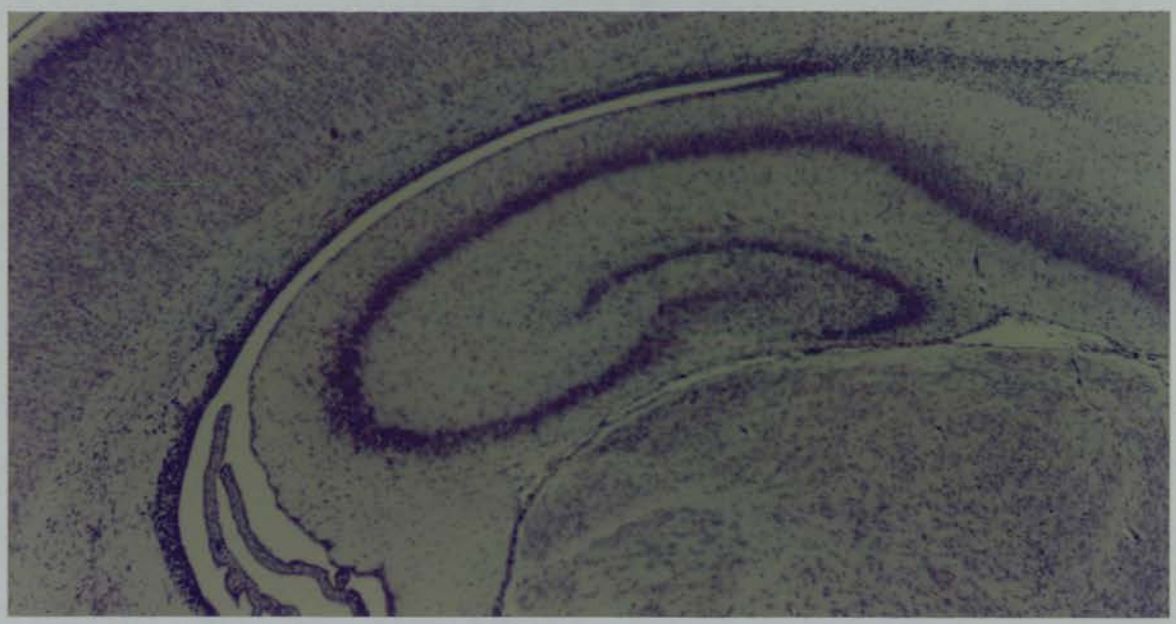

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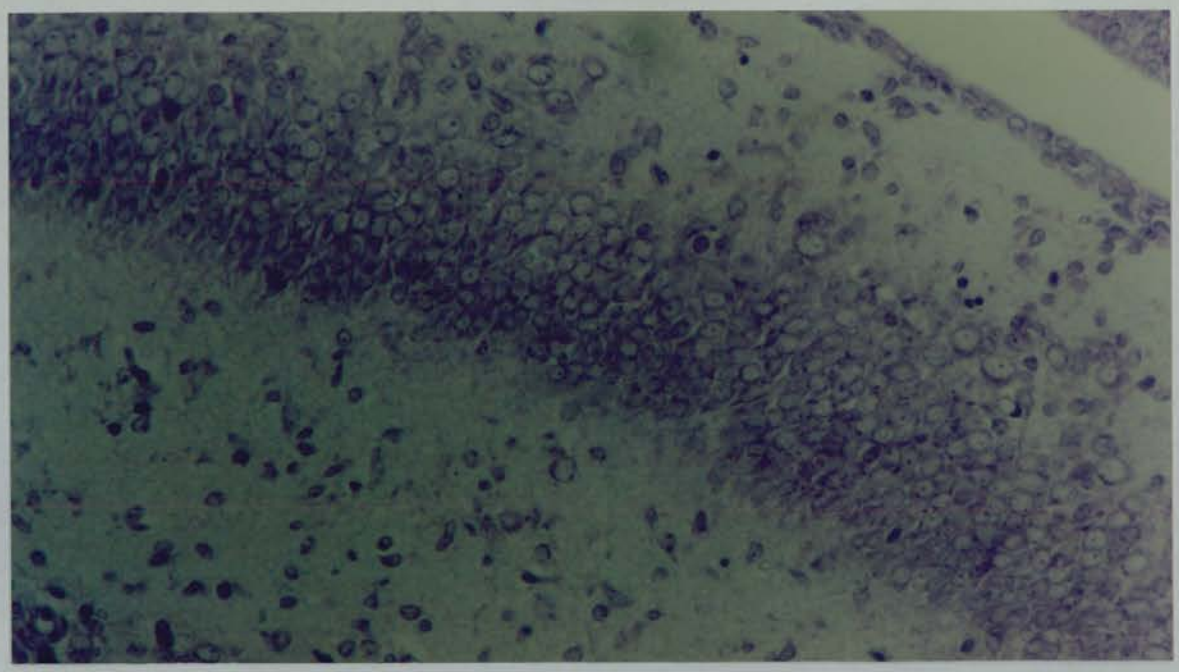

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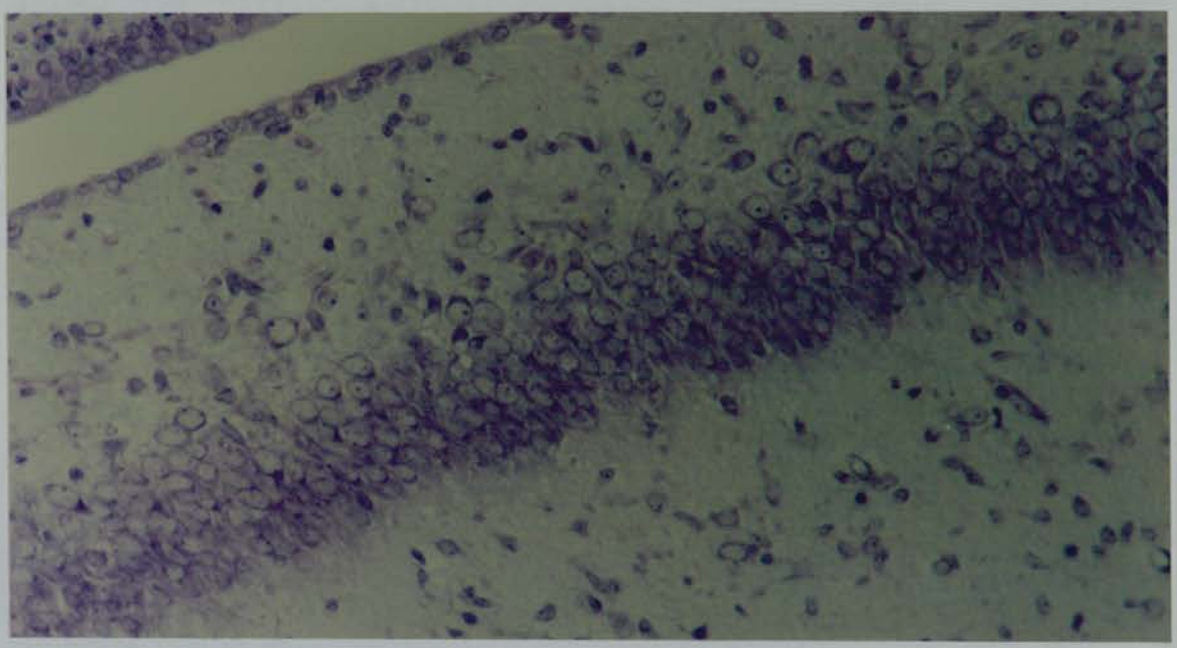

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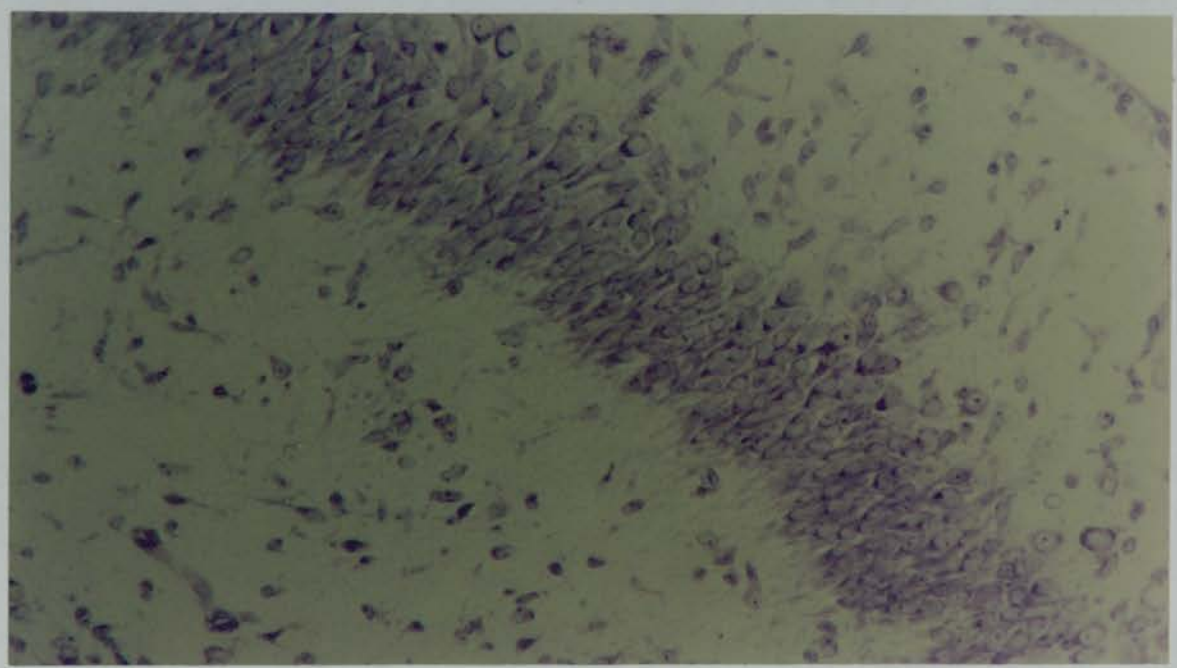

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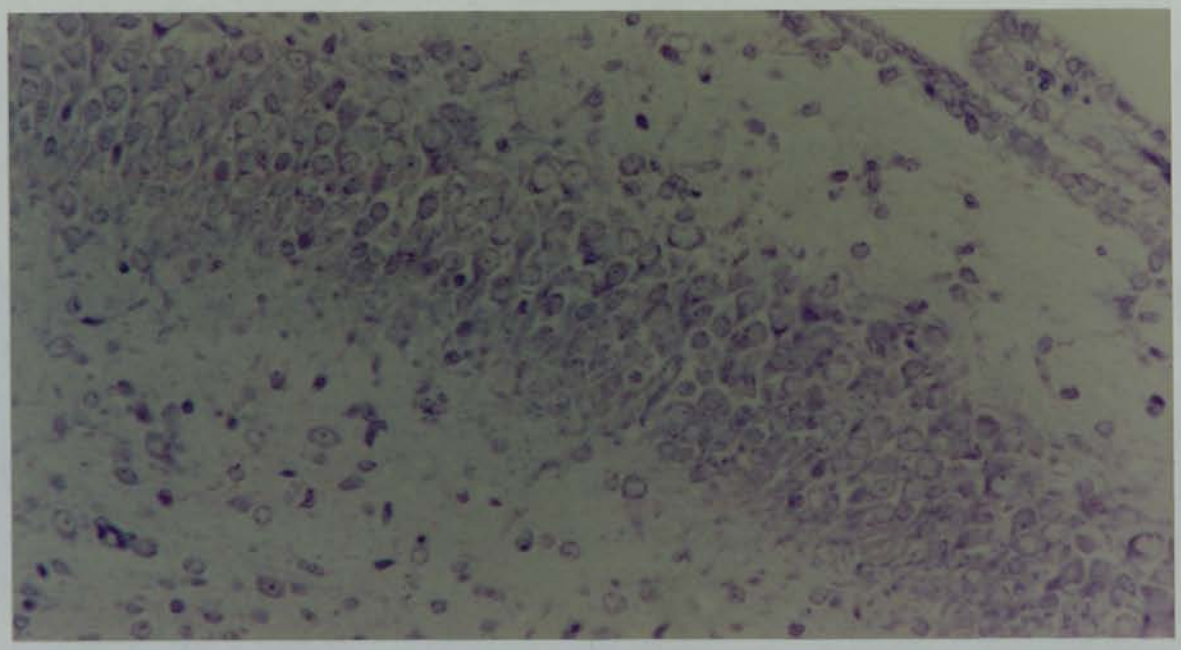

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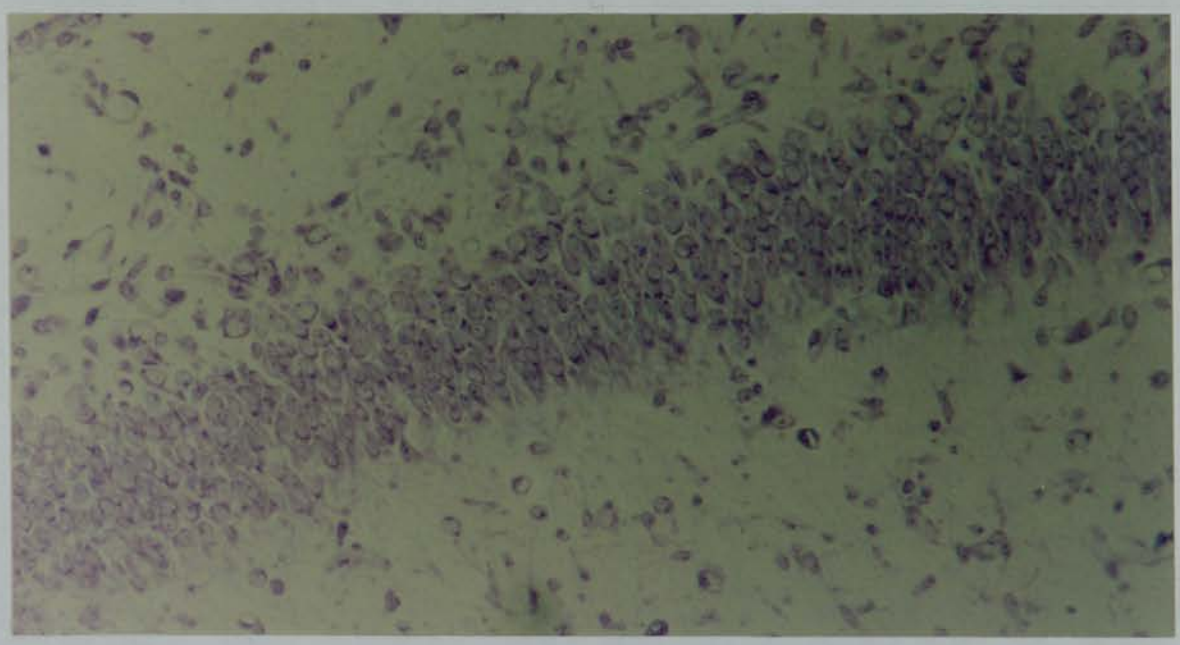

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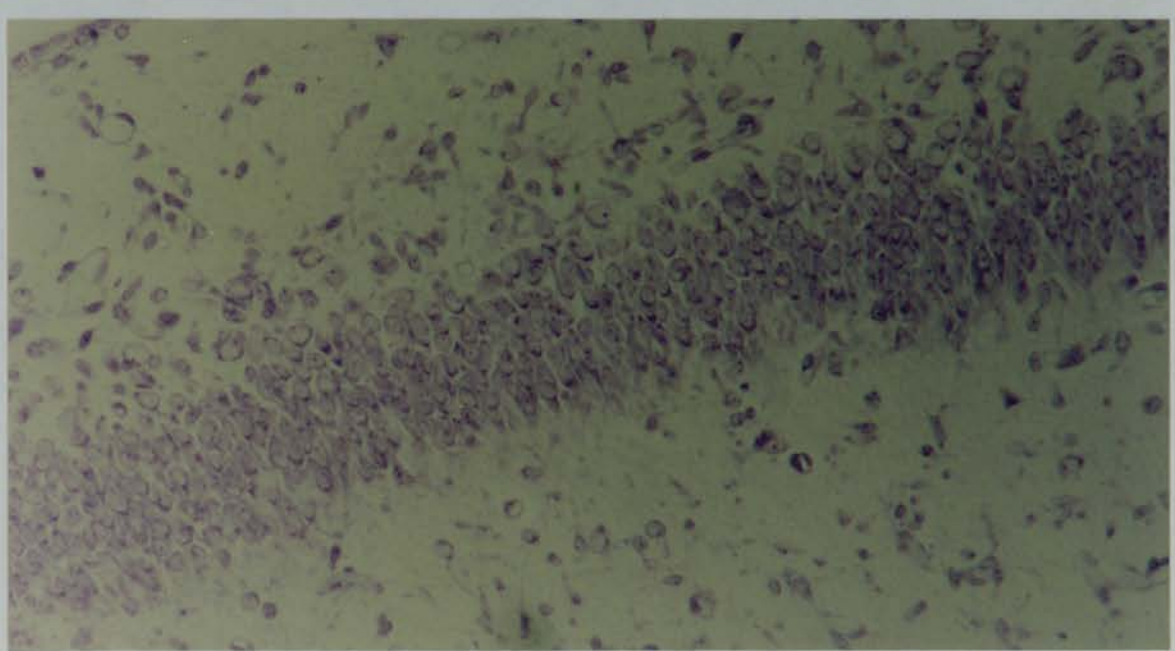

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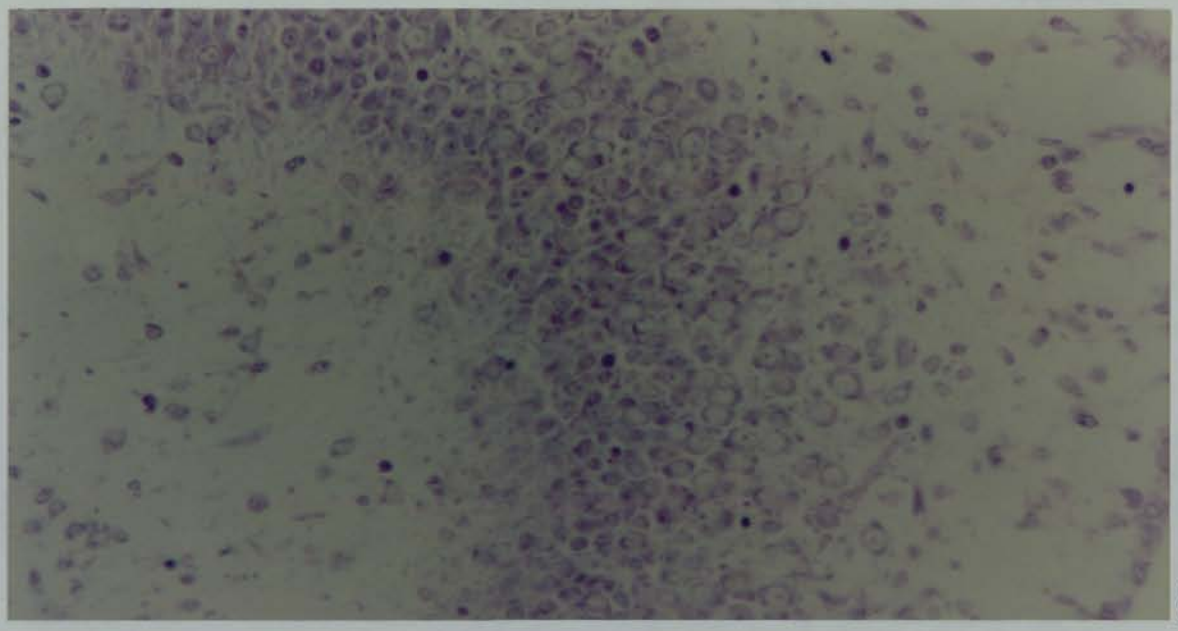

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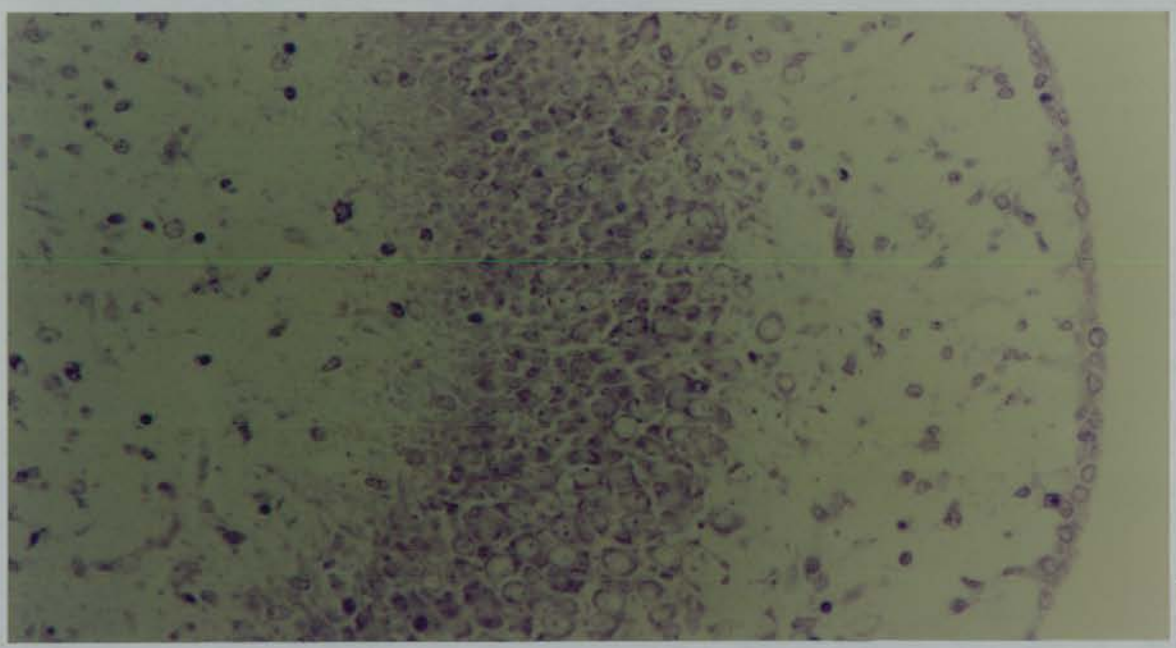

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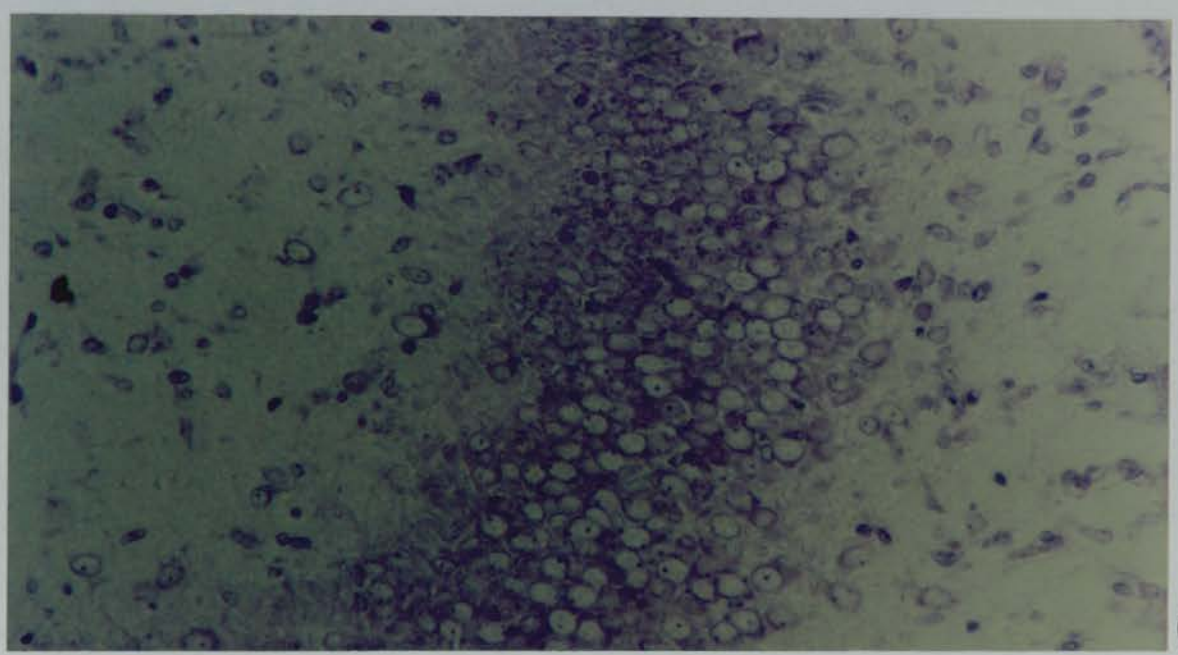

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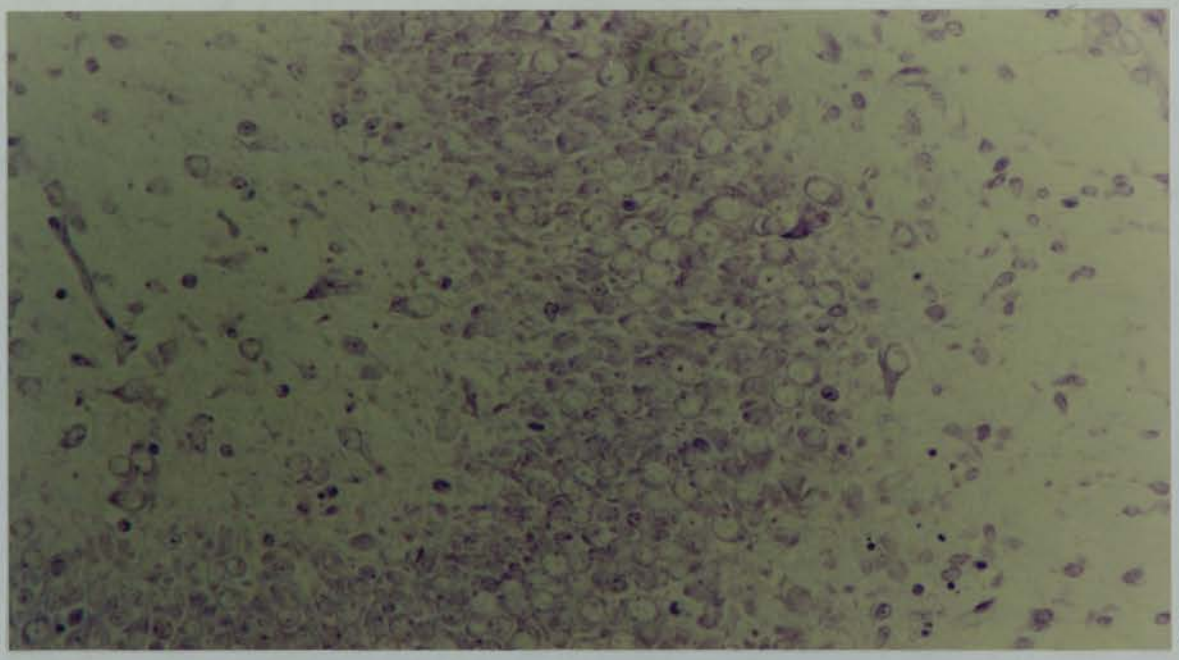

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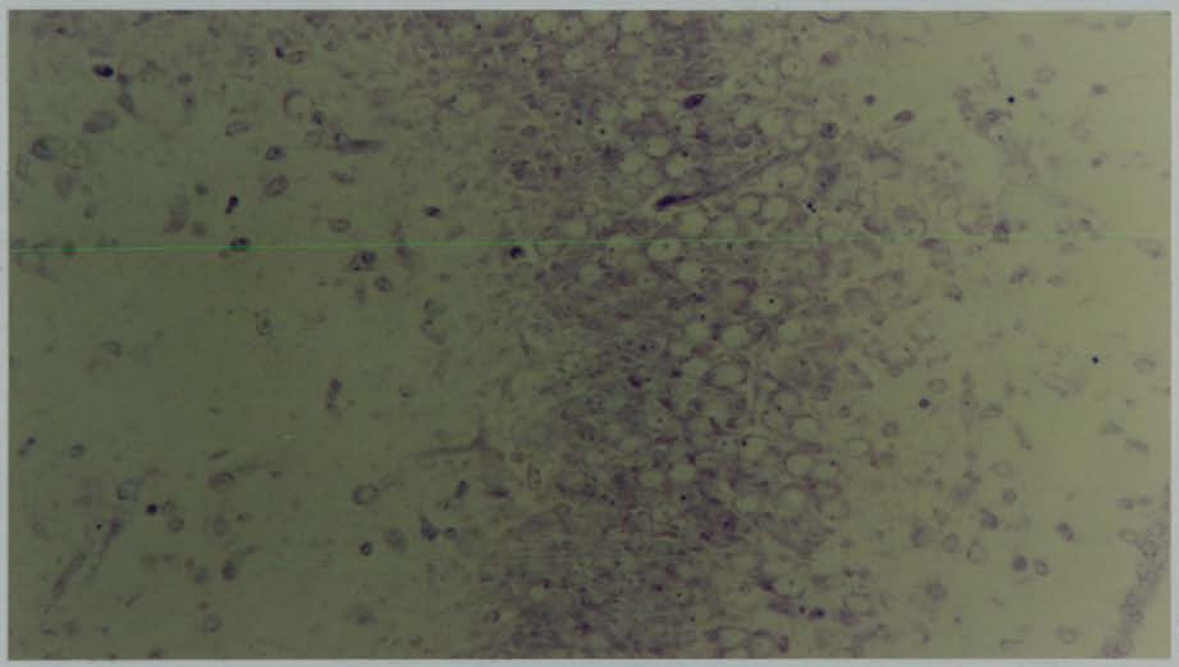

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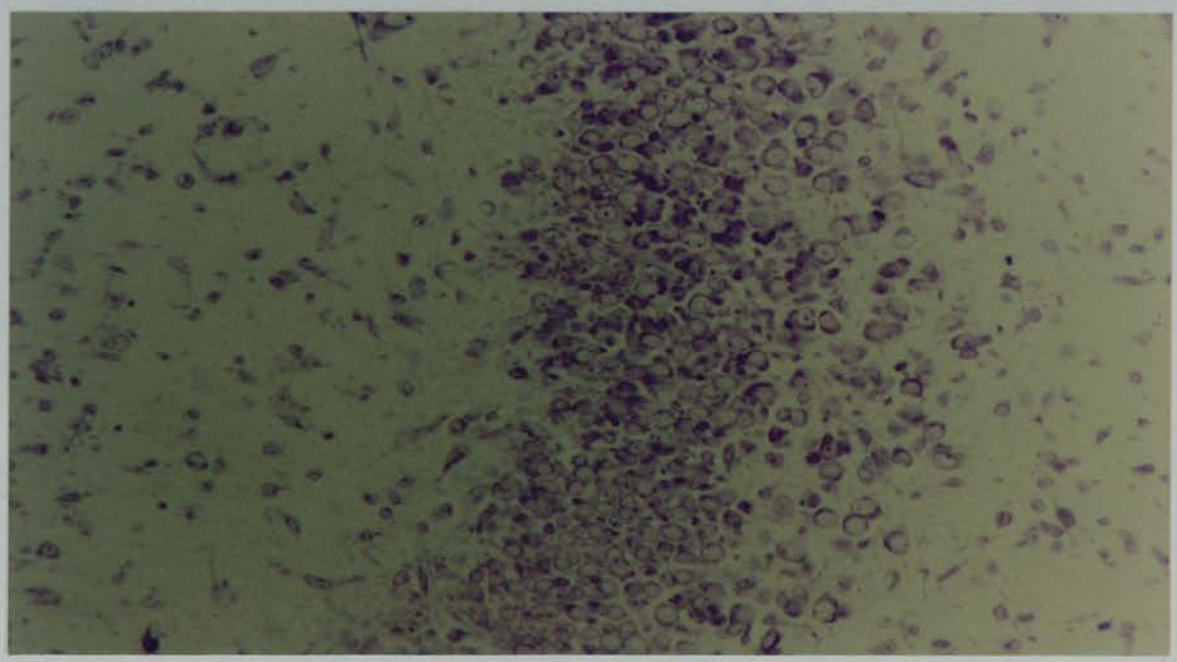

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